In vitro reproductive-endocrine, and *in vivo* immunological responses to extracted oil sands-derived naphthenic acids

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ABSTRACT

There is concern surrounding the large volumes of oil sands-influenced waters produced by the extraction of oil in the Athabasca region in northern Alberta. These waters contain water soluble organic compounds such as naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs). The goal of this study was to use *in vitro* as well as *in vivo* assays using rainbow trout (*Oncorhynchus mykiss*) combined with spectroscopic analyses to determine if specific NA fractions obtained from oil sands-influenced waters possess biological activity. NAs were extracted in bulk from oil sands-influenced waters using acid precipitation and then fractionated. NAs fractions were identified and quantified by high resolution mass spectrometry (HRMS), ^{1}H nuclear magnetic resonance (NMR), and attenuated total reflectance (ATR) infrared spectroscopy. The fraction obtained by dichloromethane (DCM) back extraction of base-solubilized showed aromatic compounds around 6.8 – 8.2 ppm using ^{1}H NMR. This fraction elicited an aryl hydrocarbon receptor (AhR)-mediated activity after 24 h at 5 mg/L. Each fraction contained a carboxylic acid dimer though there were differences in average carbon number and hydrogen deficiency between the samples. The main NA fraction was subsequently used for an *in vivo* waterborne exposure using rainbow trout. Waterborne exposures were conducted with oil sands-influenced waters, extracted NAs, and benzo[a]pyrene (BaP) as a positive control for 7 d, after which blood, spleen, head kidney, and gill samples were removed to evaluate the distribution of thrombocytes, B-lymphocytes, myeloid cells, and T-lymphocytes. The remaining trout in each experimental tank were injected with inactivated *Aeromonas salmonicida* and held in laboratory water for 21 d and subjected to similar lymphatic cell evaluation in addition to

evaluation of antibody production. Trout in the oil sands-influenced water exposure showed a decrease in B- and T-lymphocytes in blood as well as B-lymphocytes and myeloid cells in spleen and an increase in B-lymphocytes in head kidney. Because Oil sands-influenced waters affected multiple immune parameters, while extracted NAs impacts were limited, the NAs tested here are likely not the cause of immunotoxicity found in the oil sands-influenced water. Taken together, the results of spectroscopic analyses, *in vitro* assays, and *in vivo* exposures suggest that compounds capable of activating AhR-mediated pathways are present in oil sands process-affected waters, specifically in the DCM fraction. The identity of the AhR-active and immunotoxic compound(s) remains to be determined.

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LIST OF ABREVIATIONS

AhR	Aryl hydrocarbon receptor
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATR	Attenuated total reflectance
BaP	Benzo[a]pyrene
CYP1A	Cytochrome P4501A
DCM	Dichloromethane
DHT	Dihydroxytestosterone
DMSO	Dimethyl sulfoxide
DRE	Dioxin-responsive element
EDC	Endocrine disrupting chemical
ELISA	Enzyme-linked immunosorbent assays
ER	Estrogen receptor
EROD	Ethoxyresorufin-o-deethylase
ESI	Electrospray ionization
GC-MS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
Hsp	Heat shock protein
I.P.	Intraperitoneal
LC-MS	Liquid chromatography-mass spectrometry
MRM	Multiple reaction monitoring
NA	Naphthenic acid
NMR	Nuclear magnetic resonance
PAH	Polycyclic aromatic hydrocarbon
PBS/EDTA	Phosphate saline buffer/ethylenediaminetetraacetic acid
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dioxins
PCDF	Dibenzofurans
S.E.	Standard error
S.E.M.	Standard error of the mean
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF	Toxicity equivalency factor
UDP	Uridine diphosphase
YAS	Yeast androgen screen
YES	Yeast estrogen screen

CHAPTER 1

Introduction

1.1 Oil Sands Industry

The oil sands industry in north-eastern Alberta has become one of the largest crude oil producers in the world. In 2009, Alberta produced over 1.49 million barrels of crude oil/day from the oil sands industry and is projected to produce 3.2 million barrels/day by 2019 (Government of Alberta, 2011). Alberta is home to the largest known oil sands deposits, covering around 140,000 km^2 of the boreal forest (Tenenbaum et al., 2009). Approximately 27 billion m^3 of the estimated bitumen deposits can be extracted, which puts Canada's oil reserves second to Saudi Arabia's 42 billion m^3 (Weinhold, 2011). Surface mining is focused on 8 – 20 % of Alberta oil sands (FTFC, 1995a) that greatly alters the terrestrial and aquatic landscape.

Surface mined oil sands are extracted using the Clark hot water extraction method, which consumes large quantities of water primarily from the Athabasca River. Caustic soda (NaOH, pH 8) is used in the recovery of the bitumen (Rogers et al., 2002). However, this process produces large quantities of toxic water containing inorganic compounds such as clay, sand, dissolved metals, and organic compounds, including polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs). The oil sands industry is regulated under a zero discharge policy and it is for this reason that all oil sands processed water must be stored on site in large tailings pond. These tailings and treatment waters will be required to be incorporated into the reclaimed landscape in such a way that won't cause any detrimental impacts to the environment (MacKinnon and Hans Boerger,

1986). Natural or enhanced bioremediation will play an important role in detoxifying these waters by removal of toxic compounds.

1.1.1 Reclamation strategies

Reclamation strategies for oil sands disturbed areas are largely divided into two categories, those that create a wet landscape or a dry landscape. In the wet landscape approach, mined pits are partially filled with tailings and then capped with a layer of fresh water or process water to form an artificial lake (termed an "end-pit lake"). A series of experimental ponds have been utilized to examine tailings and tailings water reclamation strategies. The following experimental ponds and lakes have been used in multiple investigations found in and around the Syncrude Canada lot in Fort McMurray, Alberta and will now be summarized for comparison (Fig. 1.1). Demonstration Pond was built in 1993 using the water-capping approach, which involves the incorporation of fine tailings into the bottom of a constructed lake and adding fresh water overtop. This pond is composed of 70,000 m^3 of mature oil sands fine tailings and 70,000 m^3 of natural surface water from the muskeg area (van den Heuvel et al., 1999a). A similar pond was created at the same time as Demonstration Pond using the same mature oil sands fine tailings called Pond 10. However, this pond did not receive any natural surface water. Comparing the toxicity of both ponds will allow a better understanding of whether the water capping method is a good bioremediation technique. Previous toxicological investigations have shown that these oil sands-influenced waters have an impact on various organisms.

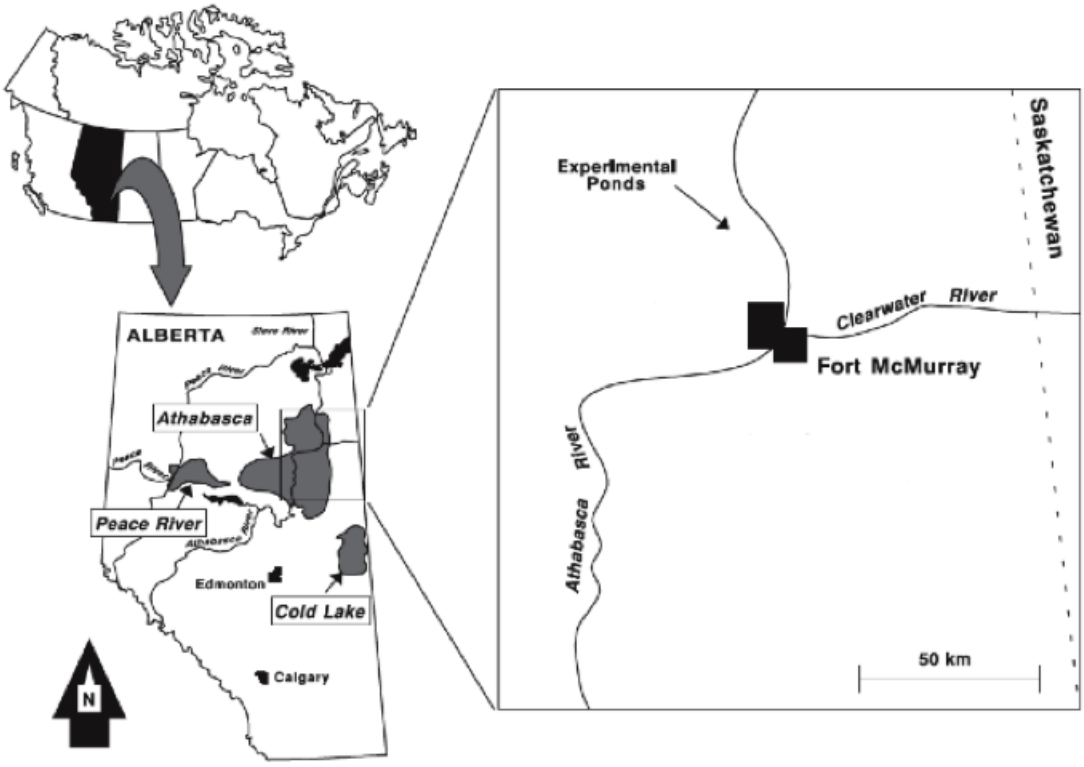

Figure 1.1 Location of experimental ponds in Fort McMurray, AB (modified from van den Heuvel et al., 1999a).

1.2 Impacts on Biota

1.2.1 Endocrine disruption

Homeostasis is the balance of physiological systems in the organism to maintain equilibrium or a relative constant steady state (Norris, 2007). The broad action of hormones, which are secreted by endocrine glands and released in the blood to affect target cells, can largely affect physiological functions. Hormones induce a response in specific tissues due to the presence of specific receptors. For example, the principal mediators of estrogen or androgen action are estrogen or androgen receptors (ER or AR). Hormone receptor binding activates the receptor, which can then stimulate an intracellular second messenger pathway or can directly bind to and stimulate regulatory gene elements. This hormone-receptor binding ultimately results in the alteration of cell function.

The interference of endocrine homeostasis by environmental pollutants is called endocrine disruption and chemicals that trigger a disturbance are called endocrine disrupting chemicals (EDCs) (Norris, 2007). Recent investigations have focused on the potential of EDCs that can mimic natural bioregulators. Of significant focus is the presence of chemicals released into the aquatic environment such as pharmaceuticals, plastics, personal care products, food, certain detergents, pesticides and mining byproducts. More recently, there is concern that oil sands mining byproducts such as oil sands-influenced waters possess endocrine activity and are capable of inducing endocrine-disrupting effects in exposed animals.

1.2.2 Endocrine disruption of oil sands-influenced waters

1.2.2.1 In vivo exposures

There is now ample evidence that the oil sands-influenced waters impact gonad size as well as cause endocrine disruption in fish. In the mid 1990's, yellow perch, indigenous to the area, were stocked in Demonstration Pond and recaptured 5 months later after which fish were examined for reproductive development and steroid production (van den Heuvel et al., 1999a, b). A similar experiment was conducted again in 2009 to compare the toxicity of these waters after a 10-year period as well as determining the effectiveness of the water capping method, which has been studied since the late 1980's (van den Heuvel et al., 2012). Male yellow perch stocked in Demonstration Pond exhibited a two-fold reduction in gonadal size between 1996 and 2009 (van den Heuvel et al., 2012). Female yellow perch also showed lower levels of plasma 17β-estradiol and testosterone in these experimental ponds (van den Heuvel et al., 1999b) as well as decreased 11-ketotestosterone (van den Heuvel et al., 2012). This unique set of experiments demonstrates the potential for oil sands-influenced waters to still affect reproductive development of fish after 10 years.

Although not indigenous to the Athabasca region, goldfish have proven to be ideal organisms for laboratory and *in situ* studies on endocrine and neuroendocrine disruption. Testosterone and 17β-estradiol levels in goldfish plasma were significantly reduced in both males and females caged for 19 d in oil sands process-affected waters (Lister et al., 2008). Another animal model, the fathead minnow, has been used both in the laboratory for controlled assessment of experimental pond water toxicity and collected from the same experimental ponds in field studies. Fathead minnow, an

indigenous species to the Athabasca region, exposed to aged tailings in the laboratory showed a decrease in testosterone in males and a decrease in 11-ketotestosterone in females (Kavanagh et al., 2011). This laboratory exposure illustrates similar results to those found in a subsequent field experiment where male fathead minnows in Demonstration Pond had lower levels of plasma 11-ketotestosterone when compared to the reference pond (Kavanagh et al., 2013). There was also an increase of gene transcripts for gonadotropin in the brain of both males and females fathead minnows exposed to West-In-Pit, an active settling basin (He et al., 2012a). Overall, endocrine effects in fish following laboratory exposures are consistent with results found in field-collected fish from oil sands impacted waters.

1.2.2.2 In vitro assessments

In vitro assays for toxicity assessment are capable of providing valuable mechanistic information in a very controlled and reproducible environment. They have often been used to assess the toxicity of contaminant-impacted waters, providing insight into mechanisms underlying *in vivo* effects previously observed in fish. However, they are also limited in that they are unable to measure the pharmacokinetics of absorption, distribution, binding, metabolism, and excretion (Freshney, 2001). An estrogen-inducible yeast cell culture developed to measure whether surfactants and their by-products have estrogenic properties (Routledge and Sumpter, 1996) has proven effective for screenings of water samples for estrogenic and androgenic activity (Brix et al., 2010; Krein et al., 2012). There are two recombinant cells line that were developed that can detect (anti-) estrogenic (YES) or (anti-)androgenic activity (YAS) in environmental samples. Thus, in

the presence of estrogen or androgen, β-galactosidase is synthesized turning the media from yellow to red (Routledge and Sumpter, 1996). Effluents from an offshore oil production platform have shown estrogenic and anti-androgenic activity with YES/YAS cells (Thomas et al., 2009) suggesting a mechanism underlying reproductive effects observed in fish exposed to oil sands-influenced waters (Kavanagh et al., 2012, 2013; Lister et al., 2008; van den Heuvel et al., 1999b, 2012). A second *in vitro* method uses the H295R cell line derived from human adrenocortical carcinoma cells that have physiological characteristics of zonally undifferentiated human adrenal cells (Hecker et al., 2006). This bioassay provides a great cost effective tool to evaluate the effects of chemicals on several steroidogenic pathways, which includes the synthesis of mineralcorticoids, glucocorticolds, androgens, and estrogens (Hecker et al., 2006). The media can then be subjected to liquid chromatography-mass spectrometry (LC-MS) or enzyme-linked immunosorbent assays (ELISA) to determine hormone production. Exposure of an H295R cell line to oil sands process-affected waters decreased production of testosterone and increased 17β-estradiol by these steroidogenic cells (He et al., 2010). The YES/YAS and H295R cell lines have seen limited use in toxicological studies involving oil sands-influenced waters, but would likely be effective screening tools for biological activity and potential for endocrine disruption. The knowledge obtained from previous laboratory and field investigations will allow researchers to dig deeper into the understanding of endocrine disrupting potential of these waters with a future wave of advanced molecular techniques.

1.2.3 Immunotoxicology

Fish immunology has received a greater level of interest in the recent years due to the potential of disease in aquaculture as well as trying to understand the health of natural populations. There are many functional and morphological similarities between mammalian and teleost leukocytes. However, fish lack hematopoietic bone marrow and identifiable lymph nodes that mammals possess. The primary location of hematopoiesis in teleosts is the head kidney as well as the thymus, spleen and mucosal tissues. The head kidney is the major producer of antibodies, which has an important role in immunological memory (Press and Evensen, 1999). Antibody-producing cells are also found in the spleen; however, the head kidney has a faster immune response and larger number of antibody-producing cells (Fänge and Nilsson, 1985).

Immunotoxicology, a subdivision within the field of toxicology, examines the immune response in maintaining integrity of the organism when exposed to a toxicant. There are a large number of chemicals known to produce immune effects by either inhibiting or enhancing responses. Chemicals that can alter an immune response of an organism include certain metals, halogenated hydrocarbons, heterocyclic compounds, organometals, and organophosphates (Iwama et al., 1996). Fish immunotoxicology is a relatively new field of study that is rapidly growing with new techniques such as specific cell staining through the use of monoclonal antibodies. Flow cytometry allows rapid multiparameter analysis of single immune cells as well as evaluates cell number and function (Chilmonczyk and Monge, 1999). These tools can then be used to determine the health of fish inhabiting oil sands-influenced waters.

1.2.4 Immunotoxicity of oil sands-influenced waters

Teleost immunocompetence in the face of environmental contaminants is important to consider when evaluating aquatic environmental health. Oil sands impacted materials and waters must be incorporated into reclaimed landscapes that support a healthy ecosystem; thus understanding the biological activity and toxicity of these waters becomes of great importance. Yellow perch exposed to waters containing elevated levels of NAs and other oil sands-related compounds in experimental ponds demonstrated an increased prevalence of the opportunistic disease lymphocystis as well as severe fin erosion (Fig. 1.2) (van den Heuvel et al., 2000; Palmer et al., 2012). Another study that caged rainbow trout in Demonstration Pond showed a decrease in total leukocyte, an increase in granulocytes and a diminished antibody response to an immune challenge (*Aeromonas salmonicida*; McNeil et al., 2012). Both studies demonstrate that oil sands-influenced waters have an impact on the immunocompetence in fish. However, the metabolic and biochemical pathways underlying these effects have yet to be determined.

Using *in vitro* cell cultures and measuring gene expression allows us to better understand the origin of the effects observed. For example, exposure of mouse bone marrow cells to a dichloromethane extract of oil sands-influenced waters and commercial NAs demonstrated a reduction in phagocytosis associated with alteration in pro-inflammatory cytokines (Garcia-Garcia et al., 2011). In a study by Wiseman et al. (2013), RNA sequencing was used to quantify abundance of transcripts in livers of male fathead minnows exposed to untreated and ozone-treated oil sands process-affected waters. The authors reported a decrease in transcripts responsible for the complement cascade in livers of fathead minnows. The down regulation of inflammatory cytokines, IFNγ, IL-

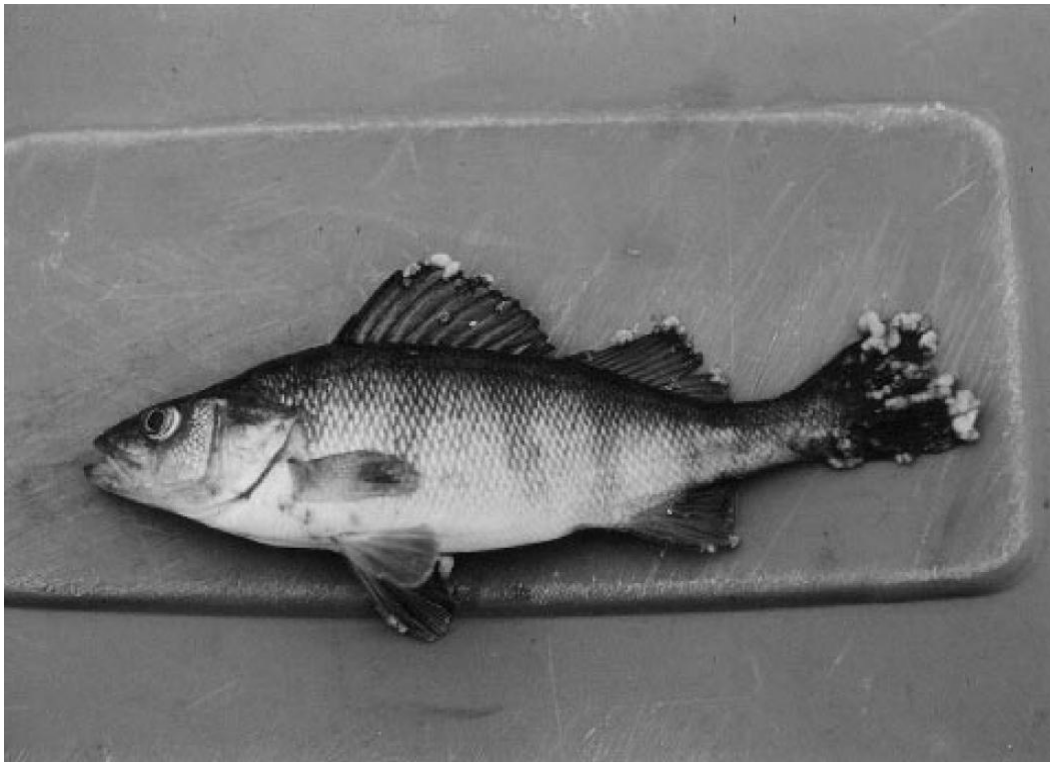

Figure 1.2 *Perca flavescens* (230 mm fork length) caught at South Bison Pond in fall 1995 with lymphocystis-like skin lesions (van den Heuvel et al., 2000), later confirmed to be lymphocystis and caused by lymphocystis disease virus (Palmer et al., 2012).

1β and CSF-1, as well as complement component reported in their study might explain the increase in lymphocystis and severe erosion found in previous field experiments.

1.2.5 Aryl hydrocarbon receptor activation

Biotransformation of chemicals is comprised of two phases that ultimately detoxifies and eliminates these compounds. Phase I either adds or exposes polar compounds while phase II reactions attempt to further increase polarity through conjugation of phase I products. The most dominant enzyme system responsible in phase I biotransformation is the cytochrome P450 (CYP) family of enzymes. CYP1 genes are induced by aromatic compounds such as PAHs, polychlorinated dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) through binding of the aryl hydrocarbon receptor (AhR). Rainbow trout have two AhR type 2 genes; AhRα and AhRβ, which have been cloned (Abnet et al., 1999). Fish AhR activity appears to have similar ligand structure-activity relationship to mammalian receptors with high affinity for TCDD binding (Hestermann et al., 2000). The AhR controls the transcription of CYP1A, CYP1A2, CYP1B, as well as phase II enzymes such as S-transferase, uridine diphosphase (UDP)-glycuronosyltransferase, and aldehyde-3-dehydrogenase (Safe, 1995). CYP1A enzyme is the CYP form most studied in toxicological assays with fish.

H4IIE-*luc* and ethoxyresorufin-o-deethylase (EROD) are two assays in which CYP1A induction is directly or indirectly measured as an indicator of AhR activation. H4IIE-*luc* cell line assays are composed of a modified rat hepatic cell line containing a luciferase reporter gene under control of a dioxin-responsive enhancer used to measure

the CYP1A induction in liver (Sanderson et al., 1996). These compounds bind to the cytosolic AhR bound to heat shock proteins (Hsp) found within H4IIE-*luc* cells. After binding, the Hsp dissociates from the complex and the activated AhR is translocated to the nucleus where it forms a dimer with the AhR nuclear translocator (ARNT). This dimer binds to specific DNA sequences, the dioxin-responsive element (DRE), which increases transcriptional activation of the luciferase reporter gene and is then read by a fluorescent plate reader (Hankinson, 1995; Hilscherova et al., 2000). Because oil sands-influenced waters contain a mixture of compounds such as aromatic compounds (Jones et al., 2012; Madill et al., 2001; Wayland et al., 2008), determining the CYP1A activity responsible for xenobiotic metabolism becomes an area of concern. However, oil sands-influenced waters did not induce AhR properties using H4IIE-*luc* cells (He et al., 2012b; Wiseman et al., 2013).

Organic pollutants from industry sources are known to induce hepatic detoxification enzymes. For example, EROD assays are used in many toxicological investigations and are often used to confirm the bioavailability and exposure to a particular organic toxicant. The post mitochondrial supernatant from the liver is isolated from fish exposed to environmental contaminants. The CYP1A enzyme catalyzes 7-ethoxyresorufin into resorufin, which can be measured fluorometrically. Liver size and EROD activity in yellow perch from Demonstration Pond and South Bison Pond was higher when compared to Mildred Lake (McNeil et al., 2012; van den Heuvel et al., 1999a and 1999b). South Bison Pond received drainage from an area reclaimed as a pasture while Mildred Lake is used as a reservoir for Athabasca River water. An increase in EROD activity was also observed in both sexes of pearl dace collected within the oil

sands deposit when compared to fish collected outside the oil sands deposit (Tetrault et al., 2003). Wood frogs exposed to young (<7 year) oil sands process-affected waters had a higher level of EROD activity when compared to an old (>7 year) oil sands process-affected waters (Hersikorn and Smits, 2011). This demonstrates that microbial activity may reduce and mitigate toxicity of certain compounds such as PAHs and NAs. These field and *in situ* studies also demonstrate the toxicity and bioavailability of aromatic compounds found in oil sands-influenced waters.

1.3 Naphthenic Acids

1.3.1 Naphthenic acid chemistry

It has been demonstrated that NAs are the primary acute toxic component of oil sands processed-affected waters (MacKinnon and Boerger, 1986). NAs are a diverse group of acyclic, monocyclic, and polycyclic carboxylic acids, with the general formula of $C_nH_{2n+z}O_2$, where n represents the carbon number and Z specifies the hydrogen deficiency resulting from ring formation or double bonds (Fig. 1.3) (Clemente and Fedorak, 2005). NAs are susceptible to environmental degradation such as anaerobic biodegradation that can modify the structure as well as the toxicity of the compound. Smaller compounds (C<22) have been shown to have higher toxicity than compounds with a higher molecular weight (C>22) (Frank et al., 2008; Holowenko et al., 2002). As well, NAs with lower molecular weight (C<17) will biodegrade much more quickly than those with a higher molecular weight (C>18) (Scott et al., 2005). Toor et al. (2013) was able to demonstrate the biodegradation of NAs by using microcosms, a simplified ecosystem, with a high hydraulic retention time of 52 weeks. During this period, NA

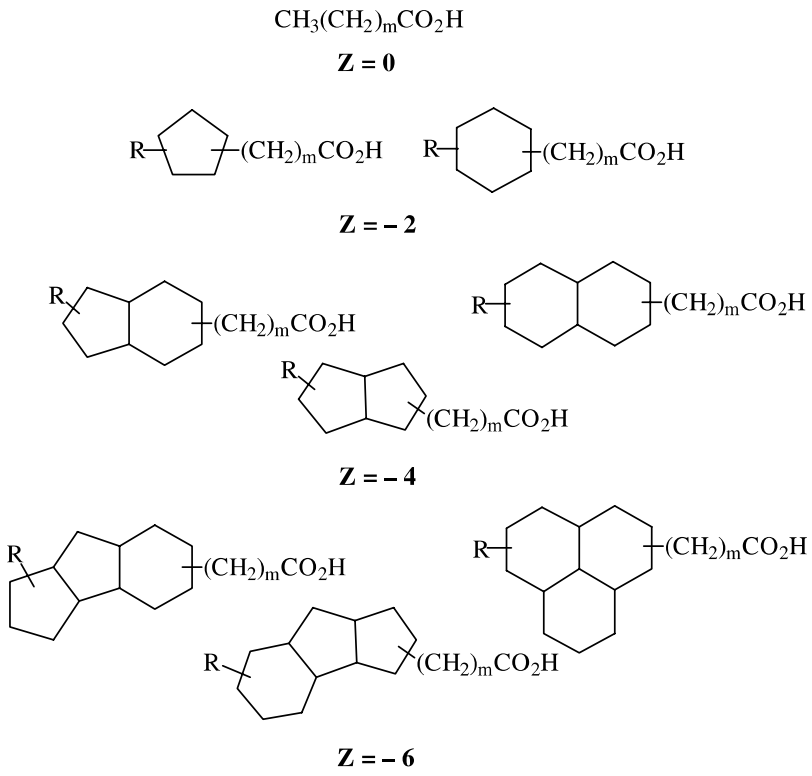

Figure 1.3 Naphthenic acid structures where m represents alkyl chain length and R represents an alkyl group (Clemente and Fedorak, 2005).

concentration was reduced by 64 – 74 % and an initial acute toxicity to rainbow trout was also reduced (Toor et al., 2013). It has been shown that there is an increase in the formation of oleic, linoleic, palmitic, and steric acids found in oil sands wastewater caused by NA degradation (Whitby, 2010; Clemente and Fedorak, 2004). These fatty acids are common constituents of prokaryotic and some eukaryotic membranes and predominate in NAs degraded by microorganisms (Whitby, 2010). The biodegradation of NAs into varied compounds may alter the toxicity of these waters; however, this remains to be determined.

Several recent studies have uncovered novel acids as part of NAs mixtures. For example, NAs mixtures also contain tricyclic diamondoid acid that are structurally different from what has been typically conceived for NAs structure (Rowland et al., 2011a and 2012). There is evidence that 'aromatic' acids can also be found in oil sands process-affected waters (Jones et al., 2012). There is also concern regarding C_{16} tetracyclic acids or isobaric pentacyclic C_{15} hydroxy acids ('O3') found in oil sands-influenced water (West et al., 2013). Uncovering these new compounds in oil sands-influenced waters is important; chemistry and spectroscopic analyses can then be used to determine the toxic modes of action of these chemicals found in these waters.

There are several studies that have been performed with commercial NAs rather than extracted NAs from oil sands tailings water. Commercial NAs generally have a lower molecular mass than the NAs found in tailings water (Scott et al., 2005), which would increase the toxicity of the mixture (MacDonald et al., 2013). Although there is some evidence that commercial NAs cause some immunotoxic effects (Hagen et al., 2012; Garcia-Garcia et al., 2011), it has been suggested that commercial mixtures may

also contain toxic components other than carboxylic acids such as C_{0-6} alkylphenols, alicyclic acids and trimethylnaphthalenes (Grewer et al., 2010; West et al., 2011). Methyl-branched, acyclic isoprenoid, cyclohexyl and isomeric octahydropentalene, perhydroindane and perhydronaphthalene acids have also been found in commercial mixtures (Rowland et al., 2011d). Because commercial mixtures contain other compounds than NAs, this confounds the typical conclusions that the classical NAs are the components responsible for toxicity.

1.3.2 Naphthenic acid toxicity

NAs exposures have demonstrated impacts on both the endocrine and immune system in fish and amphibians. Female fathead minnows exposed to extracted NAs spawned fewer eggs and males had diminished secondary sexual characteristic as well as lower levels of testosterone and 11-ketotestosterone (Kavanagh et al., 2012). The endocrine disruption results observed with extracted NAs are similar to those found in oil sands-influenced water. However, few studies have examined the immunotoxic effects of extracted NAs. After a 21 d exposure of yellow perch to extracted NAs, gill alterations included proliferation of chloride, mucous, and epithelial cells, inflammation, epithelial lifting, and structural changes to the lamellar tip fusion (Nero et al., 2006). Extracted NAs also decreased total leukocytes in rainbow trout after 5 d exposure with a 100 mg/kg intraperitoneal injection but had no effect after 21 d (MacDonald et al., 2013). This exposure caused limited effects at the highest injection dose and further investigation using environmentally relevant concentrations will need to be conducted.

Although the chemical signature of commercial vs. extracted NAs is very different, many studies have used commercial NAs when trying to elucidate mechanisms and causative agents responsible for toxicity of oil sands impacted waters. Amphibian embryos exposed to commercial NAs decreased growth and development after hatching (Melvin et al., 2012, 2013). As well, exposure to a commercial sodium naphthenate increased deformities and hatch length in yellow perch embryos (Peters et al., 2007). Immunocompetence have been observed in goldfish exposed to waterborne commercial NAs – specifically an initial stimulation of pro-inflammatory gene expression in spleen, gills, and kidney (Hagen et al., 2012). GC-MS analysis of tissue demonstrated that rainbow trout exposed to commercial NAs have a higher prevalence of NAs in gill and liver when compared to muscle and heart (Young et al., 2011). However, the exact toxicity and mechanistic behavior of commercial and extracted NAs remains to be determined.

1.4 *Oncorhynchus mykiss* as a model species

Although rainbow trout (*Oncorhynchus mykiss*) is not indigenous to the Athabasca region in north-eastern Alberta, this species is one of the best studied fish species in terms of physiology and toxicology. Rainbow trout is one of the most sensitive teleost species to environmental contaminants, particularly metals (Lacroix and Hontela, 2004). This fish is commercially available with adequate background information such as a known history of diverse sensitivities and behavioral strategies. Rainbow trout are also well adapted to laboratory studies and are an inexpensive freshwater teleost to acquire. Rainbow trout are able to cope with acute high pH (pH > 9.0) exposure through their

ammonia excretion ability, acid-base homeostasis and electrolyte balance (Wilkie et al., 1996). Monoclonal antibodies for leukcocyte differentiation have also been developed in rainbow trout and it is considered the primary model fish species for immunology and immunotoxicants.

1.5 Objectives and Hypotheses

This thesis investigates the endocrine and immune effects of NAs extracted from oil sands-influenced waters. The overarching hypothesis of this project is that NAs derived from an oil sands source causes endocrine disruption and immunotoxic effects since NAs are considered a primary toxic component of oil sands-influenced waters.

The first objective was to determine the bioactive nature of chemical fractions extracted from oil sands-influenced waters using *in vitro* bioassay methods. The first hypothesis is that the neutral compounds found in these waters will assume a planar conformation and bind to AhR to cause a CYP1A induction. The second hypothesis is that NA is responsible for endocrine disruption observed in previous investigations. To determine this, I isolated different fractions of oil sands-influenced water and measured CYP1A induction using H4IIE-*luc* cells and steroidogenic activity of these fractions using H295R cells and estrogen and androgen binding activity with YES/YAS cells. Fractions were identified and quantified by high resolution mass spectrometry (HRMS), ^{1}H nuclear magnetic resonance (NMR), and attenuated total reflectance (ATR) infrared spectroscopy.

The second objective was to determine whether oil sands-influenced waters, and oil sands-derived NAs have the potential to be immunotoxic to rainbow trout in a

laboratory exposure. The first hypothesis is that exposure to environmentally sourced water that contains oil sands-derived NAs will cause an immune suppression in the laboratory as has been observed in the field. The second hypothesis is that NAs, extracted from waters derived from the same source, will cause the same immunological effects to the pond waters.. A method was established wherein rainbow trout were exposed to oil sands-influenced waters, waterborne extracted NAs, or waterborne BaP for 7 d. The immune endpoints examined were the distribution of thrombocytes, B-lymphocytes, myeloid cells, and T-lymphocytes in blood, spleen, head kidney, and gill. Following exposures, a subset of trout were injected with formalin inactivated *A. salmonicida* and held in laboratory water for 21 d to measure antibody production. Water chemistry, NAs and BaP fluorescent bile metabolites as well as liver CYP1A induction were also determined after the 7 and 21 d exposure.

1.6 References

Abnet, C.C., Tanguay, R.L., Hahn, M.E., Heidman, W., Peterson, R.E., 1999. Two Forms of aryl hydrocarbon receptor type 2 in rainbow trout (*Oncorhynchus mykiss*). Evidence for differential expression and enhancer specificity. Journal of Biological Chemistry 274, 15159-15166.

Brix, R., Noguerol, T.-N., Piña, B., Balaam, J., Nilsen, A.J., Tollefsen, K.-E., Levy, W., Schramm, K.-W., Barceló, D., 2010. Evaluation of the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool in environmental samples. Environment International 36, 361-367.

Chilmonczyk, S., Monge, D., 1999. Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. Fish & Shellfish Immunology 9, 319-333.

Clemente, J., Fedorak, P., 2004. Evaluation of the analyses of *tert*-butyldimethylsilyl derivatives of naphthenic acids by gas chromatography-electron impact mass spectrometry. Journal of Chromatography A 1047, 117-128.

Clemente, J., Fedorak, P., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Fänge, R., Nilsson, S., 1985. The fish spleen: structure and function. Experientia 41, 152-158.

Fine Tailings Fundamentals Consortium (FTFC), 1995. Volume II: fine tails and process water reclamation. In: advances in oil sands tailings research. Alberta Department of Energy, Oil Sands and Research Division, Edmonton, AB, Canada.

Frank, R.A., Kavanagh, R., Kent Burnison, B., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R., 2008. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere 72, 1309-1314.

Freshney, I., 2001. Application of cell cultures to toxicology. Cell Biology and Toxicology 17, 213-230.

Garcia-Garcia, E., Pun, J., Perez-Estrada, L.A., Din, M.G.-E., Smith, D.W., Martin, J.W., Belosevic, M., 2011. Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. Toxicology Letters 203, 62-73.

Government of Alberta. April 2011. Talk about oil sands. [online] http://www.energy.alberta.ca/ (Accessed January 2013).

Hagen, M.O., Garcia-Garcia, E., Oladiran, A., Karpman, M., Mitchell, S., El-Din, M.G.,

Martin, J.W., Belosevic, M., 2012. The acute and sub-chronic exposures of goldfish to naphthenic acids induce different host defense responses. Aquatic Toxicology 109, 143-149.

Hankinson, O., 1995. The aryl hydrocarbon receptor complex. Annual Review of Pharmacology and Toxicology 35, 307-340.

He, Y., Wiseman, S.B., Zhang, X., Hecker, M., Jones, P.D., Gamal El-Din, M., Martin, J.W., Giesy, J.P., 2010. Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line. Chemosphere 80, 578-584.

He, Y., Patterson, S., Wang, N., Hecker, M., Martin, J.W., El-Din, M.G., Giesy, J.P., Wiseman, S.B., 2012a. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). Water Research 46, 6359-6368.

He, Y., Patterson, S., Wang, N., Hecker, M., Martin, J.W., El-Din, M.G., Giesy, J.P., Wiseman, S.B., 2012b. Toxicity of untreated and ozone-treated oil sands process affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). Water Research 46, 6359-6368.

Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R., Giesy, J.P., 2006. Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: Hormone production. Toxicology and Applied Pharmacology 217, 114-124.

Hersikorn, B.D., Smits, J.E.G., 2011. Compromised metamorphosis and thyroid hormone changes in wood frogs (*Lithobates sylvaticus*) raised on reclaimed wetlands on the Athabasca oil sands. Environmental Pollution 159, 596-601.

Hestermann, E.V., Stegeman, J.J., Hahn, M.E., 2000. Relative contributions of affinity and intrinsic efficacy to aryl hydrocarbon receptor ligand potency. Toxicology and Applied Pharmacology 168, 160-172.

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A.L., Giesy, J.P., 2000. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environmental Science and Pollution Research 7, 159-171.

Holowenko, F.M., MacKinnon, M.D., Fedorak, P.M., 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research 36, 2843-2855.

Iwama, G. K., Teruyuki, N., 1996. The immune system of fish: organism, pathogen, and environment. Academic press. San Diego, 380 p.

Jones, D., West, C.E., Scarlett, A.G., Frank, R.A., Rowland, S.J., 2012. Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract. Journal of Chromatography A 1247, 171-175.

Kavanagh, R.J., Frank, R.A., Oakes, K.D., Servos, M.R., Young, R.F., Fedorak, P.M., MacKinnon, M.D., Solomon, K.R., Dixon, D.G., Van Der Kraak, G., 2011. Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters. Aquatic Toxicology 101, 214-220.

Kavanagh, R.J., Frank, R.A., Burnison, B.K., Young, R.F., Fedorak, P.M., Solomon, K.R., Van Der Kraak, G., 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology 116-117, 34-42.

Kavanagh, R.J., Frank, R.A., Solomon, K.R., Van Der Kraak, G., 2013. Reproductive and health assessment of fathead minnows (*Pimephales promelas*) inhabiting a pond containing oil sands process-affected water. Aquatic Toxicology 130-131, 201-209.

Krein, A., Pailler, J.-Y., Guignard, C., Gutleb, A.C., Hoffmann, L., Meyer, B., Keßler, S., Berckmans, P., Witters, H.E., 2012. Determination of estrogen activity in river waters and wastewater in Luxembourg by chemical analysis and the yeast estrogen screen assay. Environment and Pollution 1, 86-96.

Lacroix, A., Hontela, A., 2004. A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch, *Perca flavescens*. Aquatic Toxicology 67, 13-21.

Lister, A., Nero, V., Farwell, A., Dixon, D.G., Van Der Kraak, G., 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Aquatic Toxicology 87, 170-177.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe, K.L., Phalen, L.J., Wagner, B.D., van den Heuvel, M.R., 2013. Immunotoxic effects of oil sands-derived naphthenic acids to rainbow trout. Aquatic Toxicology 126, 95-103.

MacKinnon, M.D., Boerger, H., 1986. Description of two treatment methods for detoxifying oil sands tailings pond water. Water Pollution Research Journal of Canada 21, 496-512.

Madill, R.E.A., Orzechowski, M.T., Chen, G., Brownlee, B.G., Bunce, N.J., 2001. Preliminary Risk Assessment of the Wet Landscape Option for Reclamation of Oil Sands Mine Tailings: Bioassays with Mature Fine Tailings Pore Water. Environmental Toxicology 16, 197-208.

McNeill, S.A., Arens, C.J., Hogan, N.S., Köllner, B., van den Heuvel, M.R., 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. Ecotoxicology and Environmental Safety 84, 254-261.

Melvin, S.D., Trudeau, V.L., 2012. Growth, development and incidence of deformities in amphibian larvae exposed as embryos to naphthenic acid concentrations detected in the Canadian oil sands region. Environmental Pollution 167, 178-183.

Melvin, S.D., Lanctôt, C.M., Craig, P.M., Moon, T.W., Peru, K.M., Headley, J.V., Trudeau, V.L., 2013. Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles. Environmental Pollution 177, 22-27.

Nero, V., Farwell, A., Lee, L., Van Meer, T., Mackinnon, M., Dixon, D., 2006. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicology and Environmental Safety 65, 252-264.

Norris, D.O., 2007. Vertebrate endocrinology. USA: Elsevier, 550 p.

Palmer, L.J., Hogan, N.S., van den Heuvel, M.R., 2012. Phylogenetic analysis and molecular methods for the detection of lymphocystis disease virus from yellow perch, *Perca flavescens* (Mitchell). Journal of Fish Diseases 35, 661-670.

Peters, L., Mackinnon, M., Van Meer, T., Van den Heuvel, M., Dixon, D., 2007. Effects of oil sands process-affected waters and naphthenic acids on yellow perch (*Perca flavescens*) and Japanese medaka (*Orizias latipes*) embryonic development. Chemosphere 67, 2177-2183.

Press, C.M., Evensen, O., 1999. The morphology of the immune system in teleost fishes. Fish & Shellfish Immunology 9, 309-318.

Rogers, V.V., Liber, K., MacKinnon, M.D., 2002. Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water. Chemosphere 48, 519-527.

Routledge, E.J. and Sumpter, J.P., 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry. 13, 241-248.

Rowland, S.J., Scarlett, A.G., Jones, D., West, C.E., Frank, R.A., 2011a. Diamonds in the rough: identification of individual naphthenic acids in oil Sands process water. Environmental Science & Technology 45, 3154-3159.

Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., 2011d. Identification of individual acids in a commercial sample of naphthenic acids from petroleum by two-dimensional comprehensive gas chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 25, 1741-1751.

Rowland, S.J., West, C.E., Scarlett, A.G., Ho, C., Jones, D., 2012. Differentiation of two industrial oil sands process-affected waters by two-dimensional gas chromatography/mass spectrometry of diamondoid acid profiles. Rapid Communications in Mass Spectrometry 26, 572-576.

Safe, S.H., 1995. Modulation of gene expression and endocrine response pathway by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. Pharmacology & Therapeutics 67, 247-281.

Sanderson, J.T., Aarts, J.M.M.J.G., Brouwer, A., Froese, K.L., Denison, M.S., Giesy, J.P., 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicology and Applied Pharmacology 137, 316-325.

Scott, A.C., MacKinnon, M.D., Fedorak, P.M., 2005. Naphthenic acids in athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environmental Science & Technology 39, 8388-8394.

Tetrault, G.R., McMaster, M.E., Dixon, D.G., Parrott, J.L., 2003. Using reproductive endpoints in small forage fish species to evaluate the effects of Athabasca oil sands activity. Environmental Toxicology and Chemistry 22, 2275-2782.

Tenenbaum, D.J., 2009. Oil sands development, a health risk worth taking? Environmental Health Perspectives 117, 150-156.

Thomas, K.V., Langford, K., Petersen, K., Smith, A.J., Tollefsen, K.E., 2009. Effect-directed identification of naphthenic acids as important *in vitro* xeno-estrogens and anti-androgens in North sea offshore produced water discharges. Environmental Science & Technology 43, 8066-8071.

Toor, N.S., Franz, E.D., Fedorak, P.M., MacKinnon, M.D., Liber, K., 2013. Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands. Chemosphere 90, 449-458.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Van Meer, T., Dobson, E.P., Dixon, D.G., 1999a. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). I. Water quality characteristics and yellow perch physiological and population responses. Canadian Journal of Fisheries and Aquatic Sciences 56, 1213-1225.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Dixon, D.G., 1999b. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). II. Chemical and biochemical indicators of exposure to oil sands related waters. Canadian Journal of Fisheries and Aquatic Sciences 56, 1226–1233.

van den Heuvel, M.R., Power, M., Richards, J., Mackinnon, M., Dixon, D.G., 2000. Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. Ecotoxicology and Environmental Safety 46, 334-341.

van den Heuvel, M.R., Hogan, N.S., Roloson, S.D., Van Der Kraak, G.J., 2012. Reproductive development of yellow perch (*Perca flavescens*) exposed to oil sands-affected waters. Environmental Toxicology and Chemistry 31, 654-662.

Weinhold, B., 2011. Alberta's oil sands, hard evidence, missing data, new promises. Environmental Health Perspectives 19, 126-131.

West, C.E., Scarlett, A.G., Pureveen, J., Tegelaar, E.W., Rowland, S.J., 2013. Abundant naphthenic acids in oil sands process-affected water: studies by synthesis, derivatisation and two-dimensional gas chromatography/high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry 27, 357-365.

West, C.E., Jones, D., Scarlett, A.G., Rowland, S.J., 2011. Compositional heterogeneity may limit the usefulness of some commercial naphthenic acids for toxicity assays. Science of The Total Environment 409, 4125-4131.

Whitby, C., 2010. Microbial naphthenic acid degradation. Advances in Applied Microbiology 70, 93-125.

Wilkie, M.P., Simmons, H.E., Wood, C.M., 1996. Physiological adaptations of rainbow trout to chronically elevated water pH (pH = 9.5). The Journal of Experimental Zoology 274, 1-14.

Wiseman, S.B., He, Y., Gamal-El Din, M., Martin, J.W., Jones, P.D., Hecker, M., Giesy, J.P., 2013. Transcriptional responses of male fathead minnows exposed to oil sands process-affected water. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 157, 227-235.

Young, R.F., Michel, L.M., Fedorak, P.M., 2011. Distribution of naphthenic acids in tissues of laboratory-exposed fish and in wild fishes from near the Athabasca oil sands in Alberta, Canada. Ecotoxicology and Environmental Safety 74, 889-896.

CHAPTER 2

In vitro assessment of endocrine disrupting potential of naphthenic acid fractions from oil sands-influenced water

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Abstract

Extraction of bitumen from oil sands produces large volumes of oil sands-influenced water and there is concern surrounding the toxicity of its constituents, especially the water-soluble organic fraction referred to as naphthenic acids (NAs). The goal of this study was to use *in vitro* assays to determine if specific NAs fractions obtained from oil sands-influenced waters possess biological activity. NAs were extracted in bulk from oil sands-influenced waters using acid precipitation. Basic aqueous extract solution was extracted with dichloromethane (DCM) to provide a neutral/hydrophobic fraction. The remaining extract was re-precipitated at acid pH of 2. Compounds that did not precipitate were extracted using C-18, resulting in two additional fractions. The four fractions were examined for aryl hydrocarbon receptor (AhR)-mediated activity using the H4IIE–*luc* bioassay, effects on production of steroid hormones by use of the H295R steroidogenesis assay, and sex steroid receptor binding activity using the yeast estrogen screen (YES) and yeast androgen screen (YAS). The chemical nature of the fractions was evaluated by high resolution mass spectrometry (HRMS), ^{1}H nuclear magnetic resonance (NMR), and attenuated total reflectance (ATR) infrared spectroscopy. The DCM fraction elicited AhR-mediated activity after 24 h at 5 mg/L, but not after 48 or 72 h, suggesting that compounds are being rapidly metabolized. Progesterone and corticosterone levels as measured in the steroidogenesis assay increased at 0.05-0.5 mg/L with the C18 MeOH fraction and 5 mg/L with the main bulk fraction, respectively. None of the fractions contained measurable levels of estrogen or androgen receptor agonists. However, a number of fractions showed anti-estrogenicity and anti-androgenicity with levels being highest in the DCM fraction. Both AhR-mediated and

anti-estrogenic/anti-androgenic receptor binding appear associated with the fraction most likely to contain neutral or hydrophobic compounds.

2.1 Introduction

In 2010, the Athabasca oil sands industry in northern Alberta reached 176 km^2 of accumulated tailings and process-affected water (CAPP, 2013). Under a zero-discharge policy, tailings and affected water must be stored on site within tailings ponds or incorporated back into a reclaimed landscape. This waste material contains particulate matter (sand and silt) as well as inorganic and organic compounds such as metals, ions, naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs).

Exposure to oil sands-influenced waters has been reported to induce a wide range of toxicological effects in fish including development, metabolism, immune function and reproduction in aquatic species, especially fishes. Studies have demonstrated decreased plasma sex steroid concentrations in yellow perch (*Perca flavescens*; van den Heuvel et al., 1999b and 2012), goldfish (*Carassius auratus*; Lister et al., 2008), and fathead minnows (*Pimephales promelas*; Kavanagh et al., 2011) exposed to aged oil sands affected-waters, as well as decreased sex steroid production in gonadal tissue from slimy sculpin (*Cottus cognatus*) collected within tributaries of the Athabasca river in the Athabasca oil sands area relative to fish from a reference site (Tetrault et al., 2003). *In vitro* studies have shown that waters from an active oil sands processed water settling basin can decrease production of testosterone and increase 17β-estradiol in a steroidogenesis assay using H295R cells (He et al., 2010) as well as affect receptor signaling (He et al., 2011).

The constituents of oil sands-influenced waters responsible for the endocrine disrupting effects are unknown, though NAs are suspected. NAs are a diverse group of compounds found in petroleum-derived materials such as crude oil. NAs are composed of acyclic, monocyclic, and polycyclic carboxylic acids, with the general formula of $C_nH_{2n+z}O_2$, where n represents the carbon number and z is zero or a negative, that specifies the hydrogen deficiency resulting from ring formation or double bonds (Clemente and Fedorak, 2005). Untransformed NAs have shown to be responsible for acute lethality in aquatic biota in oil sands-influenced water (Clemente and Fedorak, 2005; MacKinnon and Boerger, 1986). Fathead minnows exposed to extracted NAs produced from oil sands-influenced waters spawned fewer eggs and males had fewer secondary sexual traits as well as lower levels of testosterone and 11-ketotestosterone (Kavanagh et al., 2012).

The chemistry of weathered NAs in oil sands is very complex. Recent research suggests that there are aromatic acids that can be found in oil sands process-affected waters (Jones et al., 2012). Some of these aromatic compounds are structurally similar to sex steroid hormones, which have been identified as potential contaminants (Rowland et al., 2011c). Mono-oxidized ($C_nH_{2n+Z}O_3$) and di-oxidized ($C_nH_{2n+Z}O_4$) NAs were also detected in all samples of oil sands process-affected waters and in oil sands ore extracts (Han et al., 2009). Because oil sands process-affected waters contain a mixture of compounds other than NAs, such as alkyl substituted PAHs and dibenzothiophenes (Wayland et al. 2008), the biological activity of specific to NAs are difficult to assess.

The objective of this study was to use *in vitro* bioassays to determine if NAs fractions obtained from oil sands-influenced waters possessed biological activity. The

first hypothesis was that the neutral aromatic or aromatic acid compounds in the mixture would cause CYP1A induction. The second hypothesis was that NAs would elicit endocrine disrupting activity. To test these hypotheses, *in vitro* assays were used with NAs fractions to measure steroidogenic activity using H295R cells, YES/YAS assay as well as CYP1A induction using H4IIE-*luc* cells. Fractions were chemically characterized by high resolution mass spectrometry (HRMS), ^{1}H nuclear magnetic resonance (NMR), and attenuated total reflectance (ATR) infrared spectroscopy in order to relate their chemical nature to the observed biological activity.

2.2 Methods

2.2.1 Naphthenic acid extraction

NAs used in this study were extracted by acid precipitation using aged (17 year old; Pond 10) oil sands tailings water using a modified extraction method from Frank et al. (2006) and previously detailed by MacDonald et al. (2013) (Fig. 1). Briefly, water was acidified to pH 2 ± 0.2 with H_2SO_4 (Sigma, Oakville, Canada), precipitate removed and re-dissolved in pH 10 ± 0.2 with 0.1 M NaOH (Sigma). Particulate matter was removed via centrifugation of the basic solution and humic material was removed via DEAE cellulose filtration. Liquid-liquid extraction with dichloromethane (DCM) was performed to remove neutrals and the DCM was removed by nitrogen evaporation (henceforth called the 'DCM fraction'). The NAs were re-precipitated after adjustment to pH 2.0 and spun at 17,000 g for 15 min. The pellet was washed with distilled water and freeze-dried to produce a solid material (henceforth called the 'main fraction'). The supernatant was passed through a C18 cartridge and eluted with 100 % MeOH (called 'C18 MeOH'

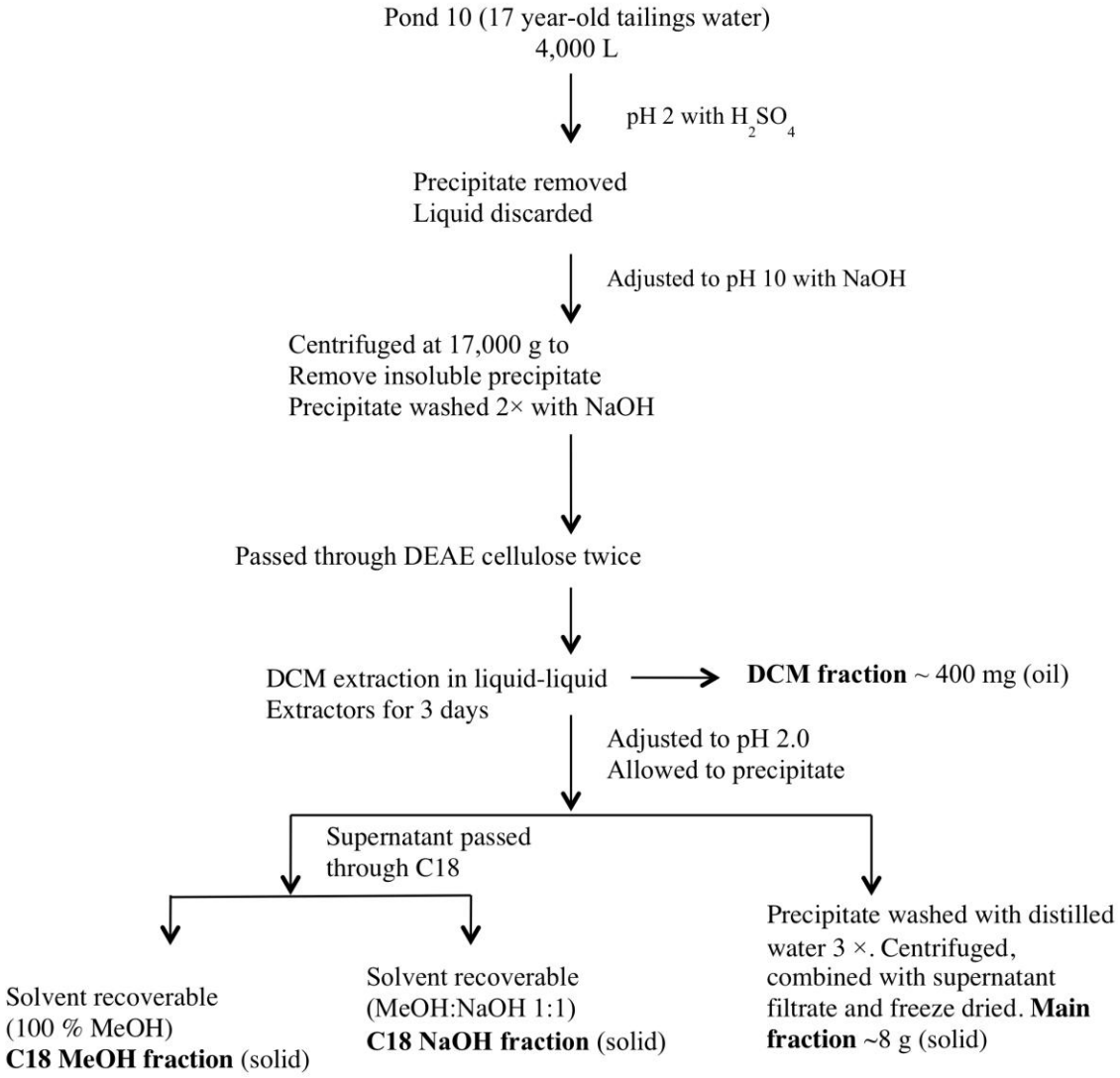

Figure 2.1 Procedure for bulk extraction and fractionation of NAs extracted from oil sands-influenced waters.

fraction) and 1:1 MeOH : 0.1 M NaOH ('C18 NaOH' fraction). Those four fractions (1) the DCM, (2) C18 MeOH, (3) C18 NaOH and (4) the main fraction were subsequently used for chemical analyses as well as *in vitro* bioassays.

2.2.2 Cytotoxicity

A cell viability test was performed to determine cytotoxic concentrations for each fraction. H4IIE-*luc* rat hepatoma cells (American Type Culture Collection (ATTC) catalog #CRL 1548) cells were propagated in DMEM/F – 12 media containing 10 % fetal bovine serum (FBS) at 37 °C, 5 % CO_2. Cytotoxicity of fractions were determined by exposing 8×10^4 H4IIE-*luc* cells to final concentrations (in the media) of 0.05 – 50 mg/L (Sigma–Aldrich, St. Louis, MO) for a period of 24 h. WST-1 reagent (Roche Applied Science, Indianapolis, IN) was used to determine metabolically active cells at the end of the incubation period according to the manufacturer's recommendations. Plates were measured using a POLARStar OPTIMA microplate reader (BMG Labtech).

2.2.3 Aryl hydrocarbon receptor transactivation assay

The assay to determine the potential for each fraction to activate a reporter gene through aryl hydrocarbon receptor (AhR) binding was conducted as previously described in Garrison et al., 1996, with modifications. The amount of AhR-induced luciferase was quantified using the LucLite(R) Reporter Gene Assay System (Perkin Elmer, Netherlands). H4IIE-*luc* cells were propagated in DMEM containing 10 % FBS at 37 °C, 5 % CO_2. Cells were plated to a concentration of 8×10^4 cells/mL in 96-well plates and incubated for 24 h. Media was removed and fractions dissolved in 0.05 % v/v, 1:1

NaOH:DMSO and subsequently dissolved in media and added to the plates. Final concentrations with media tested for each fraction range from 0.005 – 5 mg/L with time points for 24, 48 and 72 h in triplicates and two trials. Cells were harvested at each time-point and luminescence quantified using SteadylitePlus reagent (Perkin-Elmer, Waltham, MA). Luminescence derived from the exposure to fractions was compared with that obtained from a standard curve for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Wellington Laboratory, Guelph, ON) to determine TCDD equivalent concentration.

2.2.4 H295R steroidogenesis assay

The H295R steroidogenesis assay is used to determine the steroidogenic effects of test chemicals (Zhang et al., 2005; Gracia et al., 2006; Hecker and Giesy, 2011). H295R adrenal cells, purchased from ATCC (#CRL-2128; Manassas, VA), were propagated at 37 °C and 5 % CO_2 in a 1:1 mixture of DMEM/Hams F-12 medium supplemented with 2.5 % Nu-Serum (BD Biosciences, San Jose, CA, USA), 1 % ITS + Premix (BD Biosciences), and 1.2 g/L Na_2CO_3. Cells were plated in 24 well plates at a density of 3×10^5 cells/mL and incubated for 24 h. Media was removed and media containing final concentrations for each fraction range from 0.005 – 5 mg/L dissolved in 0.05 % v/v, 1:1 NaOH:DMSO were added and incubated for 48 h. Forskolin (0.1 and 10 μM) and prochloraz (0.3 and 3 μM) were used as positive controls. Media was collected and measured for testosterone, progesterone, corticosterone and androstenedione by high-performance liquid chromatography (HPLC)/mass spectrometry (MS) using an aqueous 0.1 % formic acid in nanopure and methanol gradient delivered at a flow rate of 250 μl/min. Samples were injected onto a C18 column (Betasil; Thermo Sci (part # 70105-

102130)) equipped with a C18 guard column. A triple quadruple mass spectrometer, SpectraMax 190 (Molecular Devices Corp., Sunnyvale, CA, USA) operating in positive electrospray ionization multiple reaction monitoring (MRM) mode was used to measure hormone concentrations.

2.2.5 YES/YAS

Genetically modified (recombinant) yeast (*Saccharomyces cerevisiae*) cells for detecting (anti-)estrogenic (YES) or (anti-)androgenic (YAS) activity were exposed to NA fractions. Yeast cell cultures and assays were followed according to Routledge and Sumpter (1996) and modified accordingly. Yeast cells were cultured in a 250 mL conical flask in media for 24 h at 28 °C. The fractions dissolved in 5 % NaOH in DMSO were serially diluted in 2-fold steps and 4 µL of each concentration was transferred to a 96-well flat-bottom microtiter plate in triplicate to give final concentrations in media of 0.019 – 9.9 mg/L. A YES assay containing a standard curve for 17β-estradiol for agonist standard curve of 0.027 – 54.48 µg/L, and a positive control for anti-estrogenic activity, 4-hydroxytamoxifen standard curve of 0.039 – 77.5 mg/L in the presence of 17 β-estradiol at a concentration 2.72 µg/L was conducted. A YAS assay containing a standard curve for dihydroxytestosterone (DHT) for the reference androgen agonist standard curve of 0.28 – 580.8 µg/L, and a positive control with anti-androgenic activity, 4-hydroxyflutamide 0.27 – 52.4 mg/L in the presence of DHT (29.04 µg/L) was used as androgen antagonistic control. YES standard and fractions plates tested were incubated at 38 °C for 72 h and YAS standard and fractions plates were incubated for 24 h at 38 °C and 48 h at room temperature. Plates were subsequently read on a microplate reader using

a Bio-Tek FLx800 plate reader (USA) at 550 nm and 630 nm to standardize color absorbance to relative turbidity. Absorbance derived from the exposure to fractions was compared with a standard curve for 17β-estradiol and DHT for estrogenicity and androgenicity, and curves of 4-hydroxytamoxifen and flutamide for anti-estrogenicity and anti-androgenicity to determine equivalent of the respective standards.

2.2.6 High resolution mass spectrometry

Each fraction was examined using high resolution mass spectrometry (HRMS) using a Thermo Scientific Velos Orbitrap mass spectrometer equipped through an electrospray ionization interface. The sample was infused into the mass spectrometer at a rate of 2.5 µL min^{-1}. The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in negative mode. Analyses were recorded with the highest mass resolution mode (100,000) and the observed ions $[M-H]^-$ from m/z 90 to 400 were compared with the theoretical evaluated against the predicted molecular weights of NAs according to the formula $C_nH_{2n+Z}O_2$, where z is the hydrogen deficiency (an indication of the number of rings and/or double bonds). Acid extractable structures not corresponding to the $C_nH_{2n+z}O_2$ formula are known to be present in weathered oil sands material, particularly O_3 and O_4 NAs, presumed to be alcohols of the primary NAs structures. The profile of all O_2, O_3, and O_4 NAs were evaluated for each fraction. Ratios of O_2, O_3, and O_4 NAs were derived by summing the total ion intensity of all ions within those three groups corresponding to m/z ratios of possible structures from to 5 to 30 carbons and from Z=0 to Z=-30.

2.2.7 Attenuated total resonance infrared spectroscopy

Fractions were subjected to attenuated total resonance (ATR) infrared spectroscopy to determine the functional groups found in each fraction. The data was collected at 293K on a Bruker Alpha spectrometer with an optical resolution of 0.9 cm^{-1} equipped with an Alpha-P ATR accessory using a diamond crystal. Dry samples (C18 NaOH, C18 MeOH, main) were grounded to a fine powder before applying them directly to the plate. The spectrums were then analyzed and compared for peak location and intensity.

2.2.8 Nuclear magnetic resonance

Proton nuclear magnetic resonance (NMR) characterization data were collected at 298K on a Bruker AV-300 spectrometer operating at 300.1 MHz with chemical shifts reported in parts per million (ppm) downfield of $SiMe_4$. Each fraction was dissolved in dDMSO (Cambridge Isotope, St Léonard, QC, Canada) and held in NMR tubes (5mm, Sigma). Spectra data was analyzed for peak intensities and chemical shifts.

2.2.9 Statistics

Naphthenic acid mixtures H4IIE-*luc* were quantified as their TCDD equivalent factor, (anti-)YES were evaluated as 17β-estradiol and 4-hydroxytamoxifen equivalent factor, and (anti-)YAS cell assays were evaluated for DHT, and flutamide equivalent factors. Non-linear dose vs. effect best fit curves, Y=Xmin + (Xmax-Xmin)/(1+((X/EC50)^Hillslope)), were evaluated in triplicate to determine the EC50 for each treatments on a mass basis since no molecular weight can be attributed to the

naphthenic acid mixture. The following equation was used to determine the appropriate mass equivalency factors: (EC50 standard/EC50 fraction). Prism 5 was used for these analyses.

H295R cell line assay data were analyzed by testing for normality and homogeneity of variance (Levene's and Brown-Forsythe tests) with appropriate transformations where those assumptions were not met. A full factorial two-way ANOVA comparing both trials and dose effect was performed for the H295R cell line assay followed by a post-hoc test of treatments against solvent controls using Dunnett's test. STATISTICA version 8.0 was used with an experiment-wise alpha of 0.05.

2.3 Results

2.3.1 Biological activity of fractions

The H4IIE-*luc* cytotoxicity assay showed cytotoxicity for each fraction at the highest final concentration in media of 50 mg/L. Based on the cytotoxicity assay, final concentrations in media ranging from 0.005 mg/L – 5 mg/L were subsequently used for each fraction for the H4IIE-*luc* and H295R cell assays. The DCM fraction exhibited AhR agonist activity at 5 mg/L after an incubation period of 24 h (Fig. 2.2). This corresponded to TCDD equivalency factor of 1.99×10^{-6} after 24 h and dissipated at 48 and 72 h. The remaining three fractions tested did not elicit a CYP1A induction after 24, 48 or 72 h.

Exposure to the main fraction at 5 mg/L resulted in a significant increase in corticosterone in media containing H295R cells compared to solvent control (Fig. 2.3D). Exposure to 0.05 – 0.5 mg/L of the C18 NaOH fraction resulted in greater levels of progesterone when compared to the solvent control though not in a clear dose-effect

manner (Fig. 2.3C). The DCM and C18 MeOH fractions did not alter production of any hormones.

The YES assay did not show any estrogenic effects for any of the fractions. The DCM, main, and C18 MeOH NAs fractions all showed 17β-estradiol antagonistic effects (Fig. 2.4). The 4-hydroxytamoxifen equivalent factor for those fractions was 3.7×10^{-3}, 1.2×10^{-3}, and 2.8×10^{-4} for DCM, main, and C18 MeOH fractions, respectively (Table 2.1). The YAS bioassay showed that no androgenicity was present in the fractions at the concentrations tested. The DCM and main NAs fractions showed an antagonistic effect in the YAS bioassay (Fig. 2.5). The flutamide equivalent factor for those fractions was 5.9 and 4.72 for DCM and main NAs fractions, respectively (Table 2.1).

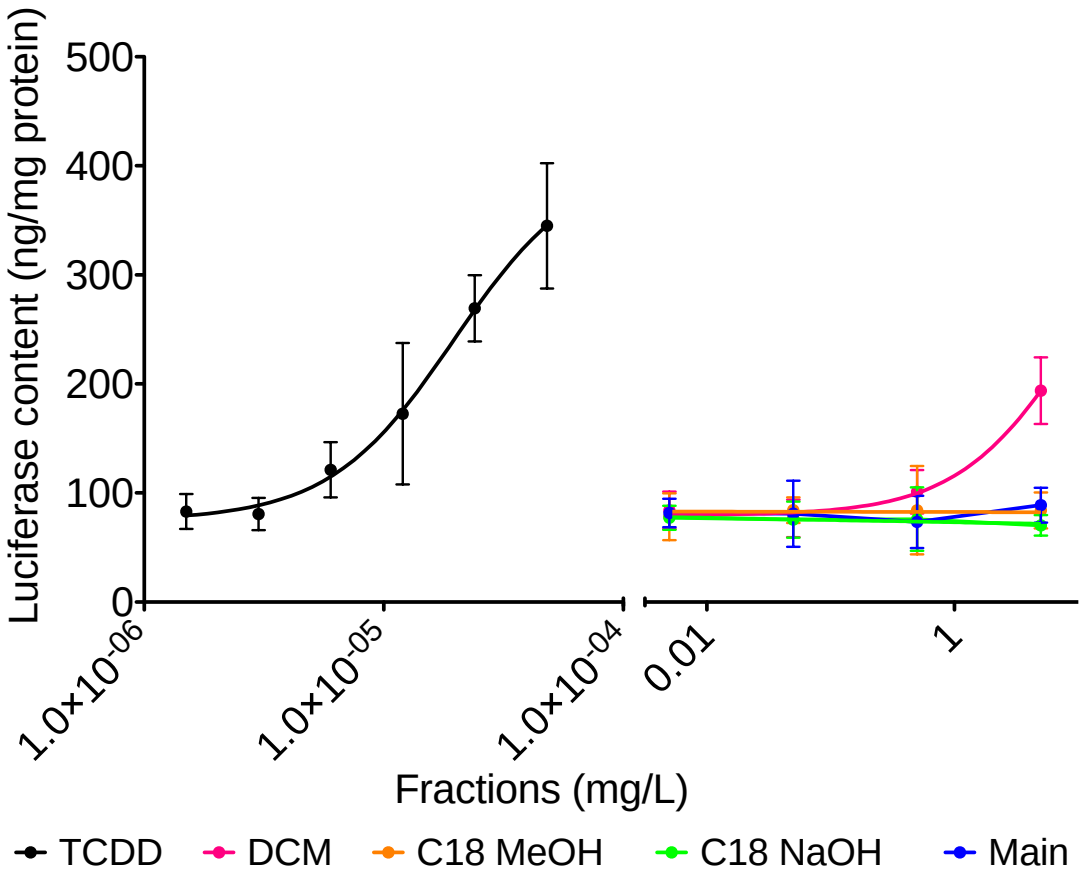

Figure 2.2 Dose-effect curve of TCDD and NA fractions using H4IIE-*luc* cells after 24 h. Samples were assayed in triplicate and two trials were conducted. Average, S.E.M. and non-linear fit curve are shown (n=2).

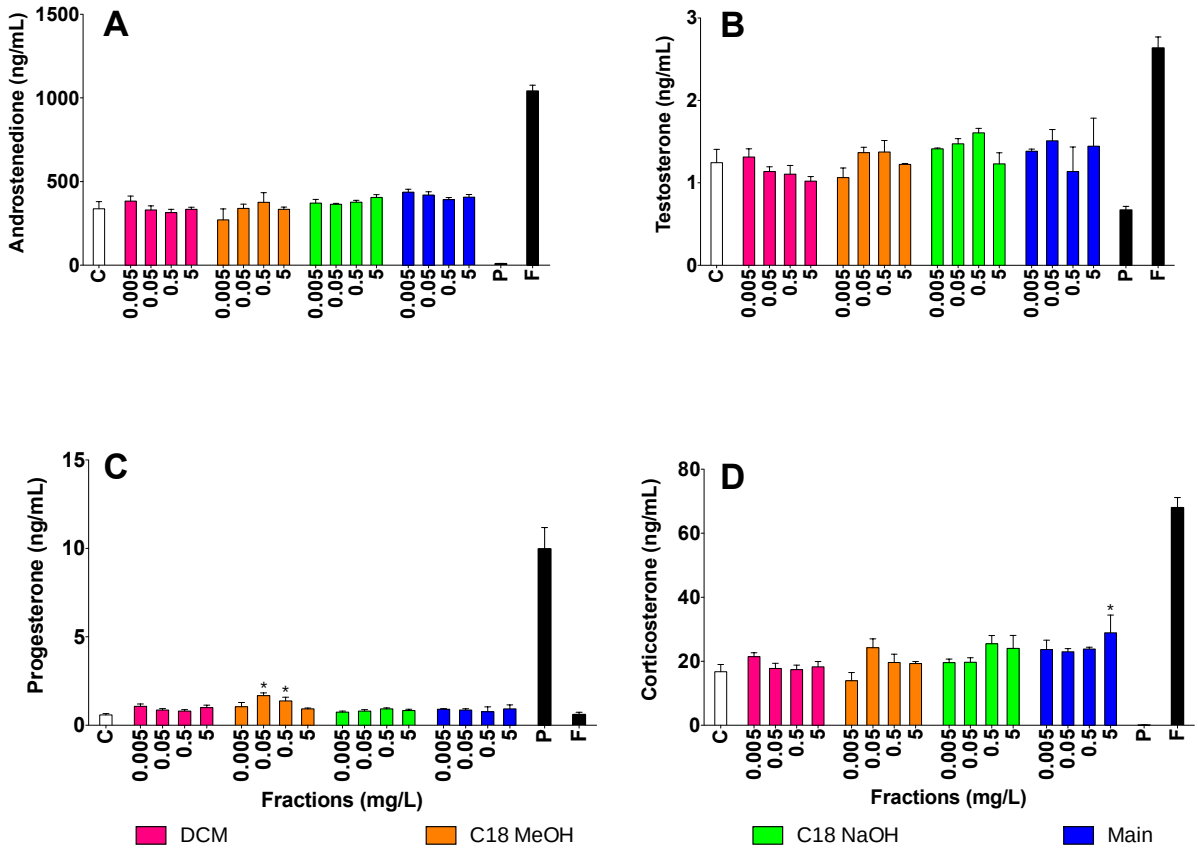

Figure 2.3 Concentration (ng/mL) of androstenedione (A), testosterone (B), progesterone (C) and corticosterone (D) in media of H295R cells exposed to NAs fractions as well as control (C), prochloraz (P) or forkoslin (F) for 48 h. Different colours indicate the NAs fractions used in the analysis. Samples were assayed in triplicate and two trials were conducted. Average and S.E.M. are shown (n=2/treatment). Asterisk indicates a significant difference between the exposed and control group within each experiment by two-way ANOVA with Dunnett's test.

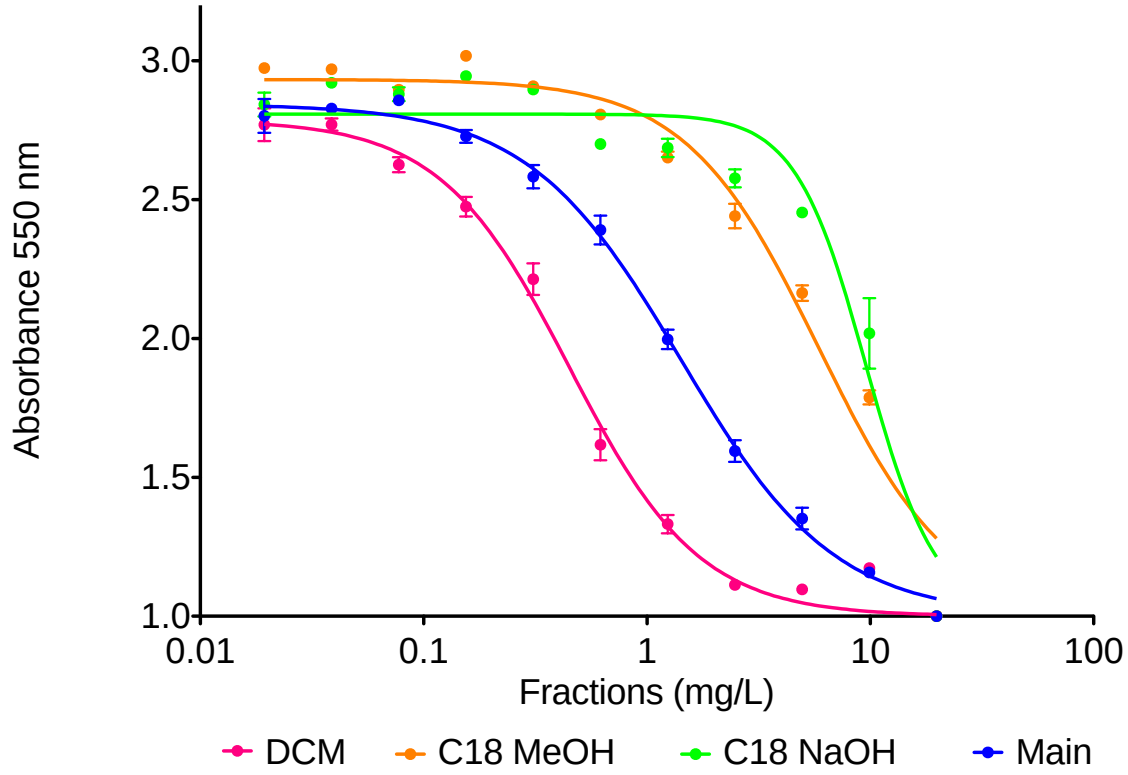

Figure 2.4 Dose-effect curve after 72 h for each NA fraction co-incubated with a constant dose of 2.72 µg/L 17β-estradiol using YES cells. NaOH:DMSO (5 % NaOH) was used as a solvent control. Average, S.E.M. and non-linear best fit curve are shown (n=3).

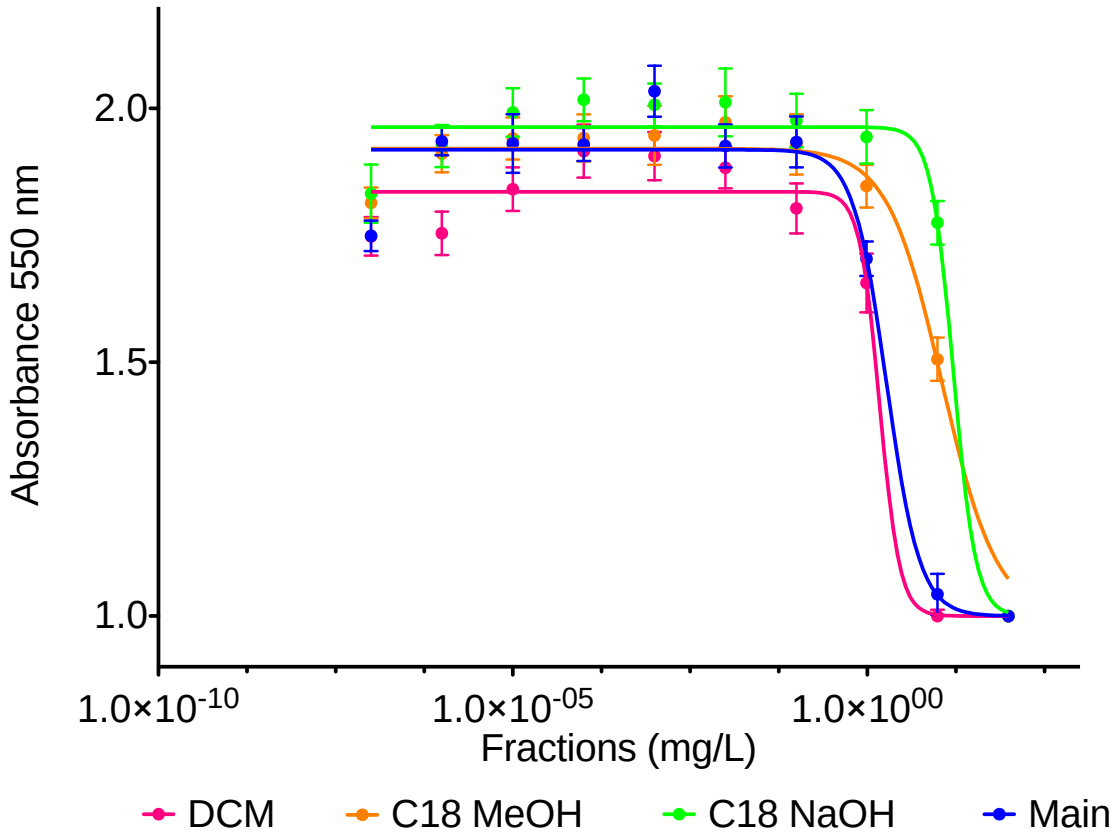

Figure 2.5 Dose-effect curve after 48 h for NA fractions with a constant dose of 29.0 µg/L dihydroxytestosterone using YAS cells . NaOH:DMSO (5 % NaOH) was used as a solvent control. Samples were assayed in duplicate and done in triplicates. Average, S.E.M. and non-linear best fit curve are shown (n=3).

Table 2.1 EC50 and TEF values for each fraction and standards for the YES, YAS and H4IIE assay.

Fractions	**EC50 (mg/L)**			**TEF**		
	YES	**YAS**	**H4IIE**	**YES**	**YES**	**H4IIE**
DCM	0.45	1.49	9.82	0.0037	5.90	1.99×10^{-6}
C18 MeOH	5.88	11.34	0	0.00028	0.78	-
C18 NaOH	9.57	16.37	0	0.00017	0.52	-
Main	1.43	1.87	0	0.0012	4.72	-
4-hydroxyTamoxifen	0.0017	-	-	-	-	-
Flutamide	-	8.81	-	-	-	-
TCDD	-	-	1.96×10^{-5}	-	-	-

2.3.2 Spectroscopic analyses

HRMS demonstrated that all fractions contained a suite of compounds with m/z ratios consistent with NAs. The C18 MeOH and C18 NaOH fraction were generally enriched in NAs with 14 or less carbons, and depleted in NAs with 15 or more carbons when compared to the main fraction (Table 2.2). As well, the C18 MeOH fraction had a higher proportion of NAO_3 and NAO_4 presumably caused by one or two additional OH groups (Table 2.2) as HRMS analysis did not show -2 charge with any of the target ions tested. Additional hydroxyl groups were also suggested by ATR infrared spectrometry by the broad shoulder peak around 3,500 – 3,200 cm^{-1} (Fig. 2.6). Each fraction demonstrated a carboxylic acid dimer and a broad carboxylic acid OH stretch signature underlying the CH bend at 1,700 cm^{-1} and 3,200 – 3,000 cm^{-1}, respectively (Fig. 2.6). ^{1}H NMR analysis of each fraction demonstrated that there was aromatic content in all of the fractions (Fig. 2.7). Unresolved peaks in the 6.8 – 7.4 ppm region suggested monocyclic aromatic moieties in all fractions. The DCM fraction had a stronger relative presence of aromatic groups at 6.8 – 8.2 ppm when compared to the main fraction (Fig. 2.6). A more complex pattern of conjugated aromatic rings is suggested by the peaks at 7.7 ppm in the DCM and main fraction only. However, based on aromatic:aliphatic proton ratios from the proton integrations, the DCM fraction had a lower relative proportion of aromatic protons than the other fractions (Table 2.2). The DCM fraction was the only fraction that had the clear presence of olefinic protons in the 5 – 6 ppm region.

Table 2.2 Average (S.E.M.) and most abundant carbon and z number for each fraction. NAs percentages demonstrate the relative concentration of each compound family, the NAs O_2, NAs with one additional oxygen (O_3), and with two additional oxygens (O_4) (charge of -1) based on parent ion intensity.

Fractions	**Average**		**Most abundant**		**NA (%)**			**Aromatic : aliphatic ratio**
	Carbon	**Z number**	**Carbon**	**Z number**	**O_2**	**O_3**	**O_4**	
C18 MeOH	12.62 (0.23)	-3.75 (0.20)	14	-4	88.08	9.79	2.14	1:4.6
C18 NaOH	13.08 (0.27)	-4.08 (0.07)	14	-4	96.06	3.37	0.57	1:2.3
DCM	15.28 (0.27)	-4.38 (0.06)	16	-4	96.42	2.97	0.61	1:21.6
Main	15.35 (0.34)	-5.33 (0.24)	15	-4	99.82	0.15	0.03	1:9.0

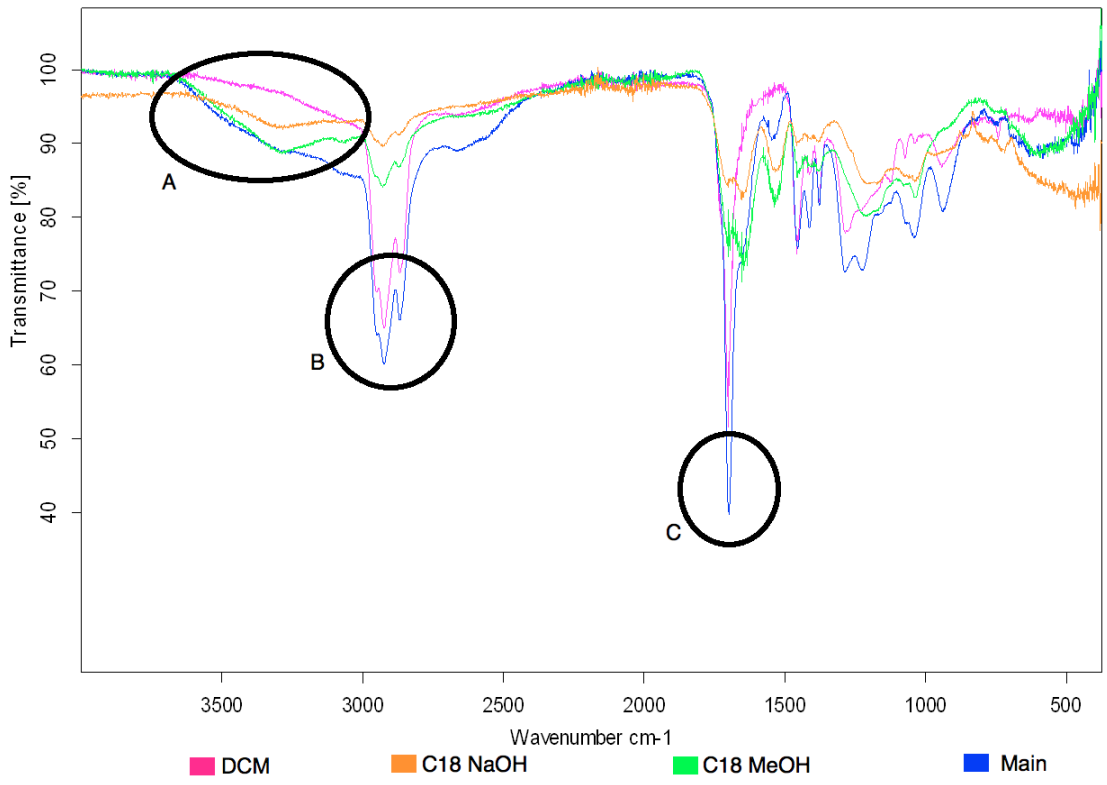

Figure 2.6 ATR infrared spectroscopy of four NAs fraction extracted from oil sands process-affected waters. Different colours indicate the NAs fractions used in the analysis. (A) hydroxyl groups, (B) CH bend, and (C) carboxylic acid dimer.

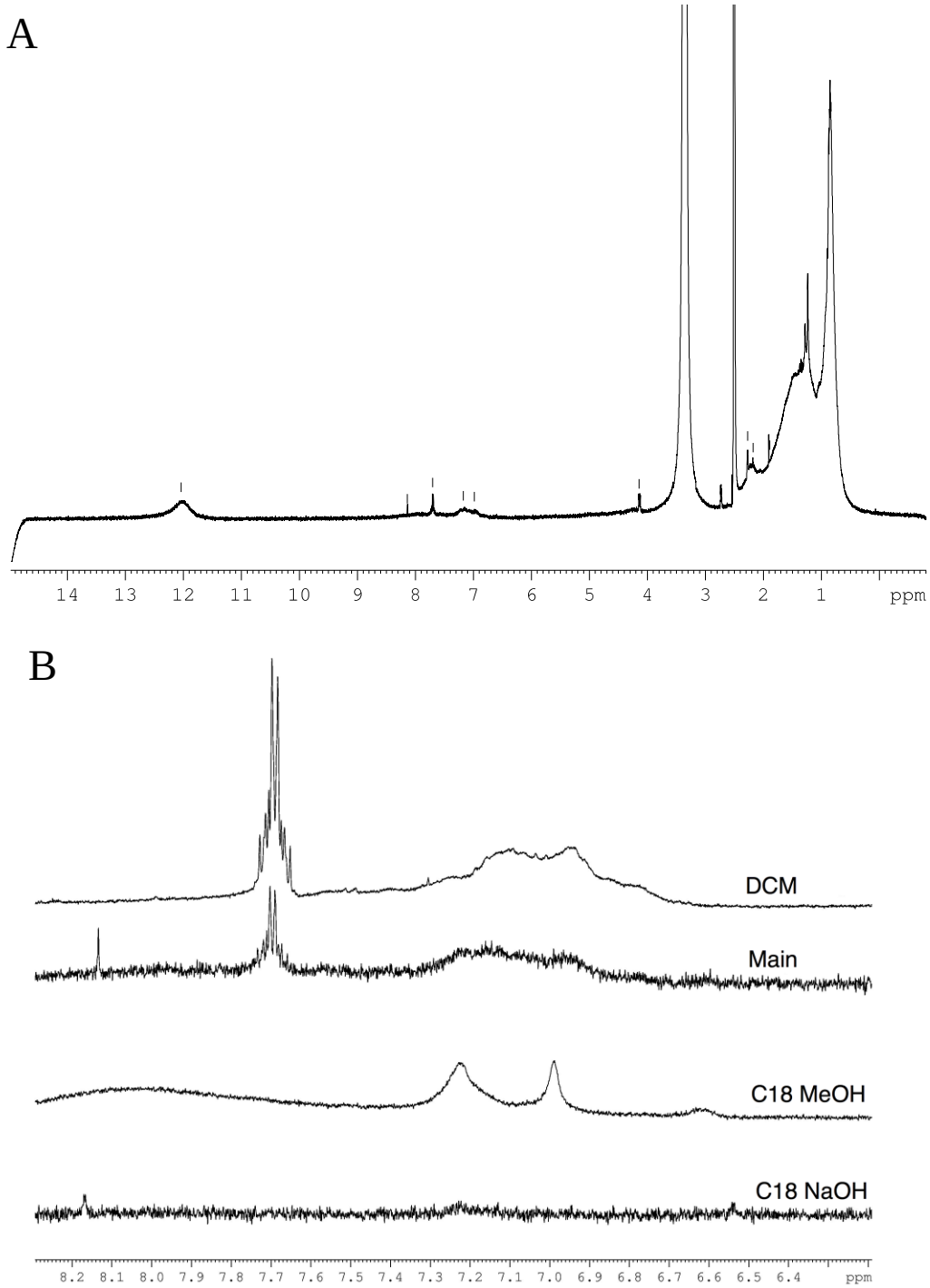

Figure 2.7 ^{1}H NMR of the main fraction (A) and a comparison of four NAs fraction (B) extracted from oil sands process-affected waters. Spectra was centered on the aromatic region for comparison.

2.4 Discussion

The present study describes the *in vitro* screening of aged NA extract fractions in order to identify biological activity associated with each fraction. Simulation of corticosterone production in H295R cells was demonstrated by the main fraction. NA fractions did not show measurable levels of estrogenic or androgenic compounds. However, anti-estrogenic and anti-androgenic activity was found in most of the fractions, and was highest in the DCM fraction. Only the DCM fraction was found to contain AhR agonists. Chemical analysis indicated that these results for anti-estrogenicity, anti-androgenicity and AhR agonism were not consistent with increased aromaticity of extracted fractions according to proton NMR. In contrast, those fractions had no influence on steroidogenesis, though stimulatory effects were observed with the main fraction.

Neutral compounds present in the DCM fraction could likely be the cause of the AhR induction observed in H4IIE-*luc* cells after 24 h of exposure. Planar compounds such as polycyclic aromatic hydrocarbons (PAHs), biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) induce CYP1A activity. Because the induction attenuated after 48 and 72 h, this suggests that these compounds found in this fraction are labile, being rapidly metabolized. While NMR suggests that the active fraction was not more enriched in aromatic moieties, IR shows that this fraction was still dominated by NAs, and neutrals such as PAH may have comprised only a small component of the mixture. In previous studies employing the same assay, West-In-Pit, an active settling basin, did not induce AhR properties (He et al., 2012b; Wiseman et al., 2013). However, the AhR agonist compounds found in the DCM fraction are highly concentrated from

4,000 L of aged tailings water, and enriched in that fraction, a fraction that represents only about 4 % of the total extracted material. The H4IIE-*luc* results are consistent with the CYP1A induction in fishes exposed to tailings-influenced waters of the same origin (Arens et al., 2013; McNeil et al., 2012; van den Heuvel et al., 1999b, 2012). Also consistent with the H4IIE-*luc* results was that the main NAs fraction used here also failed to induce CYP1A in rainbow trout as measured by EROD activity in previous studies (Chapter 3; MacDonald et al., 2013).

AhR inducing compounds found in the DCM fraction are suggestive of a structure containing at least two benzene rings. Based on the ^{1}H NMR spectra, the two benzene rings are likely not conjugated, which would have a spectrum higher than 8 ppm. The peak present around 7.7 ppm is consistent with compounds such as dibenzothiophenes, or other heterocycles. Dibenzothiophenes and heterocyclic aromatic compounds have been used in previous investigations as simple analogs of organic S found in petroleum materials (Kannel and Gan, 2012). Dibenzothiophenes, heterocycles, and PAHs have previously been found in oil sands tailings pond in sediments (Madill et al., 2001) as well as in insect larvae and adult insects (Wayland et al., 2008). The pond water used in the NAs extraction was 17 years old and it has previously been estimated that NAs undergo slow biodegradation with a half-life in the range of 12.8 – 13.6 years (Han et al., 2009). This would alter the structure, size, as well as biological activity of NAs compounds found in these waters. For example, the South American heavy crude oil demonstrated around 100 homologues containing O_2, O_2S, O_3S, O_4, and O_4S heteroatoms (Qian and Robbins, 2001), similar to those found here. The presence of hydroxy acids (O_3 compounds) has also been reported in oil sands process-affected water in northern

Alberta (West et al., 2013). The study at hand also identified oxidized NAs and showed that they are removed from the main NAs fraction by the extraction process. Despite rapid degradation, other NAs compounds remain recalcitrant for decades and it could be speculated that diamondoid-type structures could be resistant to bacterial degradation.

The larger NAs found in the main fraction caused an increase in corticosterone. The increase of corticosterone might be linked to immunosuppression observed in previous investigations (Chapter 3; MacDonald et al., 2013; McNeil et al., 2012; van den Heuvel et al., 2000). In a previous study, rainbow trout were exposed to the main NAs fraction via intraperitoneal injection, resulting in increased leukocytes in blood and a mild inflammatory response (MacDonald et al., 2013). There was also a significant decrease in thrombocytes in spleen after a 7 d waterborne exposure to the main NAs fraction (Chapter 3). This indicates that immunocompetence of rainbow trout to NAs might be linked to the increase in corticosterone seen in the main fraction. Stress hormones have detrimental effects on immune function, including lymphocyte populations and lymphocyte proliferation (Webster Marketon and Glaser, 2008). Similar results have been demonstrated when southern toads (*Bufo terrestris*) exposed to coal combustion waste had higher levels of corticosterone and testosterone when compared to the reference site (Hopkins et al., 1997). The possible relationship of stress induced immunotoxicity remains uncertain as this fraction was not shown to be a potent immunotoxicant in rainbow trout (Chapter 3).

The main NAs fraction contains aromatic compounds as demonstrated by the low intensity peak at 7.7 ppm. A previous investigation showed that extracted NAs may include aromatic compounds such as alicyclic and acyclic naphtheno-aromatic acidic

compounds measured by GC × GC-MS (Jones et al., 2012) and we suspect that these are the compound represented by the peak found at 7.7 ppm. Using the $C_nH_{2n+z}O_2$ general formula, the mass spectra of the main NAs fraction is comprised mainly of C12 – 19 bi-tetracyclic acids. Oil sands process-affected waters have been previously reported to show tricyclic and bicyclic acids as the major components (Headley et al., 2009; Rowland et al., 2011b). Recent advances in NAs characterization using comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCxGC/ToF-MS) has allowed researchers to more accurately identify individual compounds found in oil sands process-affected waters (Rowland et al., 2011a, b, 2012; West et al., 2013). Compounds found in these waters and most likely to be found in the main NAs fraction include diamantane, ring-opened diamantane carboxylic acid, methyl esters and sulfur-containing species.

The lower molecular weight and more water soluble C18 fractions showed little biological activity with the exception of some weak anti-estrogenicity. These fractions contained a higher proportion of NAO_3 and NAO_4 when compared to the DCM and main fraction. The NAO_3 and NAO_4 compounds would be less likely to precipitate with the addition of H_2SO_4 due to the addition of one or two hydroxyl or ether groups. The smaller molecular weight of the C18 fractions would also have contributed to their higher water solubility. These compounds would remain in the supernatant and subsequently be retained by the C18/18 cartridges and eluted with methanol. The compounds found in this fraction do not have two carboxylic acids because by using HRMS, these compounds would show a -2 ionization, which was not found. These results were measured by HRMS negative-ion mode, which allows acidic compounds such as NAs to be readily

observed under these conditions as well as O_x compounds (Barrow et al., 2010).

The anti-estrogenic and anti-androgenic activity found may explain previously observed reproductive effects of oil sands-influenced waters. The only other direct *in vitro* examination of these biological activities evaluated NAs from offshore oil platforms. In contrast to the present study, this study found the mixtures to be mildly estrogenic and anti-androgenic (Thomas et al., 2009). However, components of that work were conducted with commercial sources of NAs that are dissimilar to the mixtures used herein. Previous investigations have shown that fathead minnows had lower levels of testosterone when exposed to oil sands process-affected waters (Kavanagh et al., 2011) and extracted NAs (Kavanagh et al., 2012). Oil sands process-affected waters also decreased in testosterone in exposed H295R cell lines (He et al., 2010). Female yellow perch inhabiting oil sands-influenced waters also showed lower plasma steroid levels (van den Heuvel et al., 1999b, van den Heuvel et al., 2012). Plasma testosterone and 17β-estradiol levels in goldfish were significantly reduced in both males and females caged for 19 d in oil sands process-affected waters (Lister et al., 2008). However, the extracted NAs fractions did not have an impact on testosterone levels using H295R cells but did induce anti-androgenic activity in YAS cells at concentrations lower than what would be found in oil sands-influenced waters. This demonstrates that the yeast assay is an effective tool for screening of water samples for estrogenic and androgenic activity as seen in previous investigations (Brix et al., 2010; Krein et al., 2012). Steroidal aromatic NAs (Rowland et al., 2011c) as well as alkylphenols (Jones et al., 2012; West et al., 2011) have been previously reported in the Athabasca oil sands process-affected waters. These compounds might be responsible for the effects observed in YES/YAS cells.

However, the biological activity of these specific compounds responsible for these effects has not been determined.

The results of the present study demonstrate that extracted NAs fractions demonstrate both AhR activity and reproductive-endocrine disruption. The anti-androgenic and anti-estrogenic impacts of extracted NAs are similar to those observed in previous investigations (Lister et al., 2008; Kavanagh et al., 2011; van den Heuvel et al., 1999b, 2012). Dibenzothiophenes and heterocyclic aromatic compounds remain likely compounds responsible for this induction.

2.5 References

Arens, C.J., Hogan, N.S., Van Der Kraak, G.J., van den Heuvel, M.R., 2013. Sublethal effects of oil sands-affected water on white sucker (*Catostomus commersonii*). Environmental Toxicology and Chemistry submitted.

Barrow, M.P., Witt, M., Headley, J.V., Peru, K.M., 2010. Athabasca oil sands process water: characterization by atmospheric pressure photoionization and electrospray ionization fourier transform ion cyclotron resonance mass spectrometry. Analytical Chemistry 82, 3727-3735.

Brix, R., Noguerol, T.-N., Piña, B., Balaam, J., Nilsen, A.J., Tollefsen, K.-E., Levy, W., Schramm, K.-W., Barceló, D., 2010. Evaluation of the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool in environmental samples. Environment International 36, 361-367.

Canadian Association of Petroleum Producers, 2013. The facts on: Oil sands.

Clemente, J., Fedorak, P., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Frank, R.A., Kavanagh, R., Burnison, B.K., Headley, J.V., Peru, K.M., Der Kraak, G.V., Solomon, K.R., 2006. Diethylaminoethyl-cellulose clean-up of a large volume naphthenic acid extract. Chemosphere 64, 1346-1352.

Garrison, P.M., Tullis, K., Aarts, J.M.M.J., Brouwer, A., Giesy, J.P., Denison, M.S., 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. Toxicological Sciences 30 (2), 194–203.

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Zhang, X., Hecker, M., Higley, E.B., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2006. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicology and Environment Safety 65 (3), 293–305.

Han, X., MacKinnon, M.D., Martin, J.W., 2009. Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS. Chemosphere 76, 63-70.

He, Y., Wiseman, S.B., Zhang, X., Hecker, M., Jones, P.D., Gamal El-Din, M., Martin, J.W., Giesy, J.P., 2010. Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line. Chemosphere 80, 578-584.

He, Y., Wiseman, S.B., Hecker, M., Zhang, X., Wang, N., Perez, L.A., Jones, P.D., Gamal El-Din, M., Martin, J.W., Giesy, J.P., 2011. Effect of ozonation on the

estrogenicity and androgenicity of oil sands process-affected water. Environmental Science & Technology 45, 6268-6274.

He, Y., Wiseman, S.B., Wang, N., Perez-Estrada, L.A., El-Din, M.G., Martin, J.W., Giesy, J.P., 2012a. Transcriptional responses of the brain–gonad–liver axis of fathead minnows exposed to untreated and ozone-treated oil sands process-affected water. Environmental Science & Technology 46, 9701-9708.

He, Y., Patterson, S., Wang, N., Hecker, M., Martin, J.W., El-Din, M.G., Giesy, J.P., Wiseman, S.B., 2012b. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). Water Research 46, 6359-6368.

Hecker, M., Giesy, J.P., 2011. Effect-Directed Analysis of Ah-Receptor Mediated Toxicants, Mutagens, and Endocrine Disruptors in Sediments and Biota. 15, 285-313.

Headley, J.V., Peru, K.M., Barrow, M.P., 2009. Mass spectrometric characterization of naphthenic acids in environmental samples: A review. Mass Spectrometry Reviews 28, 121-134.

Hopkins, W.A., Mendonc, M.T., Congdon, J.D., 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. General and Comparitive Endocrinology 108, 237-246.

Jones, D., West, C.E., Scarlett, A.G., Frank, R.A., Rowland, S.J., 2012. Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract. Journal of Chromatography A 1247, 171-175.

Kannel, P.R., GAN, T.Y., 2012. Naphthenic acids degradation and toxicity mitigation in tailings wastewater systems and aquatic environments: A review. Journal of Environmental Science and Health, Part A 47, 1-21.

Kavanagh, R.J., Frank, R.A., Burnison, B.K., Young, R.F., Fedorak, P.M., Solomon, K.R., Van Der Kraak, G., 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology 116-117, 34-42.

Kavanagh, R.J., Frank, R.A., Oakes, K.D., Servos, M.R., Young, R.F., Fedorak, P.M., MacKinnon, M.D., Solomon, K.R., Dixon, D.G., Van Der Kraak, G., 2011. Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters. Aquatic Toxicology 101, 214-220.

Krein, A., Pailler, J.-Y., Guignard, C., Gutleb, A.C., Hoffmann, L., Meyer, B., Kebler, S., Berckmans, P., Witters, H.E., 2012. Determination of estrogen activity in river waters and wastewater in Luxembourg by chemical analysis and the yeast

estrogen screen assay. Environment and Pollution 1, 86-96.

Lister, A., Nero, V., Farwell, A., Dixon, D.G., Van Der Kraak, G., 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Aquatic Toxicology 87, 170-177.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe, K.L., Phalen, L.J., Wagner, B.D., van den Heuvel, M.R., 2013. Immunotoxic effects of oil sands-derived naphthenic acids to rainbow trout. Aquatic Toxicology 126, 95-103.

MacKinnon, M.D., Boerger, H., 1986. Description of two treatment methods for detoxifying oil sands tailings pond water. Water Pollution Research Journal of Canada 21, 496-512.

Madill, R.E.A., Orzechowski, M.T., Chen, G., Brownlee, B.G., Bunce, N.J., 2001. Preliminary Risk Assessment of the Wet Landscape Option for Reclamation of Oil Sands Mine Tailings: Bioassays with Mature Fine Tailings Pore Water. Environmental Toxicology 16, 197-208.

McNeill, S.A., Arens, C.J., Hogan, N.S., Köllner, B., van den Heuvel, M.R., 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. Ecotoxicology and Environmental Safety 84, 254-261.

Qian, K., Robbins, W.K., 2001. Resolution and identification of elemental dompositions for more than 3000 crude acids in heavy petroleum by negative-ion microelectrospray high-field fourier transform ion cyclotron resonance mass spectrometry. Energy & Fuels 15, 1505-1511.

Routledge, E.J. and Sumpter, J.P. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry*. 13: 241-248.

Rowland, S.J., Scarlett, A.G., Jones, D., West, C.E., Frank, R.A., 2011a. Diamonds in the rough: identification of individual naphthenic acids in oil sands process water. Environmental Science & Technology 45, 3154-3159.

Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., Frank, R.A., 2011b. Identification of individual tetra- and pentacyclic naphthenic acids in oil sands process water by comprehensive two- dimensional gas chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 25, 1198–1204.

Rowland, S.J., West, C.E., Jones, D., Scarlett, A.G., Frank, R.A., Hewitt, L.M., 2011c. Steroidal aromatic 'naphthenic acids' in oil Sands process-affected water: structural comparisons with environmental estrogens. Environmental Science & Technology 45, 9806-9815.

Rowland, S.J., West, C.E., Scarlett, A.G., Ho, C., Jones, D., 2012. Differentiation of two

industrial oil sands process-affected waters by two-dimensional gas chromatography/mass spectrometry of diamondoid acid profiles. Rapid Communications in Mass Spectrometry 26, 572-576.

Tetrault, G.R., McMaster, M.E., Dixon, D.G., Parrott, J.L., 2003. Using reproductive endpoints in small forage fish species to evaluate the effects of Athabasca oil sands activity. Environmental Toxicology and Chemistry 22, 2275-2782.

Thomas, K.V., Landford, K., Petersen, K., Smith, A.J., Tollefsen, K.E., 2009. Effect-directed identification of naphthenic acids as important *in vitro* xeno-estrogens and anti-androgens in North Sea offshore produced water. Environmental Science and Technology 43, 8066-8071.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Dixon, D.G., 1999b. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). II. Chemical and biochemical indicators of exposure to oil sands related waters. Canadian Journal of Fisheries and Aquatic Sciences 56, 1226-1233.

van den Heuvel, M.R., Power, M., Richards, J., Mackinnon, M., Dixon, D.G., 2000. Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. Ecotoxicology and Environmental Safety 46, 334-341.

van den Heuvel, M.R., Hogan, N.S., Roloson, S.D., Van Der Kraak, G.J., 2012. Reproductive development of yellow perch (*Perca flavescens*) exposed to oil sands-affected waters. Environmental Toxicology and Chemistry 31, 654-662.

Wayland, M., Headley, J.V., Peru, K.M., Crosley, R., Brownlee, B.G., 2008. Levels of polycyclic aromatic hydrocarbons and dibenzothiophenes in wetland sediments and aquatic insects in the oil sands area of Northeastern Alberta, Canada. Environmental Monitoring and Assessment 136, 167-182.

Webster Marketon, J.I., Glaser, R., 2008. Stress hormones and immune function. Cellular Immunology 252, 16-26.

West, C.E., Jones, D., Scarlett, A.G., Rowland, S.J., 2011. Compositional heterogeneity may limit the usefulness of some commercial naphthenic acids for toxicity assays. Science of The Total Environment 409, 4125-4131.

West, C.E., Scarlett, A.G., Pureveen, J., Tegelaar, E.W., Rowland, S.J., 2013. Abundant naphthenic acids in oil sands process-affected water: studies by synthesis, derivatisation and two-dimensional gas chromatography/high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry 27, 357-365.

Wiseman, S.B., He, Y., Gamal-El Din, M., Martin, J.W., Jones, P.D., Hecker, M., Giesy, J.P., 2013. Transcriptional responses of male fathead minnows exposed to oil sands process-affected water. Comparative Biochemistry and Physiology Part C:

Toxicology & Pharmacology 157, 227-235.

Zhang, X., Yu, R.M.K., Jones, P.D., Lam, G.K.W., Newsted, J.L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, J.T., Wu, R.S.S., Giesy, J.P., 2005. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environmental Science and Technology 39 (8), 2777–2785.

CHAPTER 3

The Immunological Effects of Oil Sands Surface Waters and Naphthenic Acids on Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

There is concern surrounding the immunotoxic potential of naphthenic acids (NAs), a major organic constituent in waters influenced by oil sands contamination. To assess the immunological response to NAs, rainbow trout (*Oncorhynchus mykiss*) waterborne exposures were conducted with oil sands-influenced waters, NAs extracted and isolate from oil sands tailings waters, and benzo[a]pyrene (BaP) as a positive control. After a 7 d exposure, blood, spleen, head kidney, and gill samples were removed from a subset of fish in order to evaluate the distribution of thrombocytes, B-lymphocytes, myeloid cells, and T-lymphocytes using fluorescent antibodies specific for those cell types coupled with flow cytometry. The remaining trout in each experimental tank were injected with inactivated *Aeromonas salmonicida* and held in laboratory water for 21 d and subjected to similar lymphatic cell evaluation in addition to evaluation of antibody production. Fluorescent metabolites in bile as well as liver CYP1A induction were also determined after the 7 and 21 d exposure. Oil sands waters and extracted NAs exposures resulted in an increase in bile fluorescence at phenanthrene wavelengths, though liver CYP1A was not induced in those treatments as it was with the BaP positive control. Trout in the oil sands-influenced water exposure showed a decrease in B- and T-lymphocytes in blood as well as B-lymphocytes and myeloid cells in spleen and an increase in B-lymphocytes in head kidney. The extracted NAs exposure showed a decrease in thrombocytes in spleen at 8 mg/L and an increase in T-lymphocytes at 1 mg/L in head kidney after 7 d. There was a significant decrease in antibody production against *A. salmonicida* in both oil sands-influenced water exposures. Because oil sands-influenced waters affected multiple immune parameters, while extracted NAs impacts

were limited, the NAs tested here are likely not the cause of immunotoxicity found in the oil sands-influenced water.

3.1 Introduction

In 2009, Canadian bitumen production averaged 1.49 million barrels/day from the oil sands industry and is projected to produce 3.2 million barrels/day by 2019 (Government of Alberta, 2011). The Clarke hot water extraction method used to recover bitumen from oil sands requires about 3 m^3 of water for about 4 m^3 of fluid tailings (Holowenko et al., 2002). Industry is required to safely incorporate resultant tailings and tailings water back into the reclaimed landscape. However, there is concern that substances associated with oil sands mining will enter the wider environment and cause detrimental effects on biota such as teleost fish (Kelly et al., 2009). The tailings and wastewaters contain clay, sand, dissolved metals, and organic compounds, including polycyclic aromatic hydrocarbon (PAH) and naphthenic acid (NA).

NAs are a diverse group of compounds found in petroleum-derived materials such as crude oil. NAs are composed of acyclic, monocyclic, and polycyclic carboxylic acids, with the general formula of $C_nH_{2n+z}O_2$, where n represents the carbon number and Z specifies the hydrogen deficiency resulting from ring formation or double bonds (Clemente and Fedorak, 2005). Recent research suggests that NAs mixtures contain tricyclic diamondoid acids that differ from what has been generally conceived for NAs structure (Rowland et al., 2011; 2012). The complexity of NAs is further increased as they become transformed in the environment. The more saturated and lower molecular weight NAs tend to be eliminated entirely through rapid biodegradation, whereas the

larger more complex NAs may not be mineralized, but can be oxidized to other structures such as alcohols (Han et al., 2009). Due to the chemical complexity, commercially available NAs mixtures do not at all resemble the chemical profile of oil sands-derived NAs in the environment (MacDonald et al., 2013).

It has been demonstrated that untransformed NAs are responsible for acute lethality in aquatic biota (MacKinnon and Boerger, 1986). However, little is known regarding whether untransformed or transformed NAs are involved in chronic toxicity observed upon exposure to oil sands-influenced waters. Yellow perch (*Perca flavescens*) exposed to waters containing elevated levels of NAs and other oil sands-related compounds in experimental ponds demonstrated an increased prevalence of opportunistic diseases including lymphocystis and severe fin erosion (Palmer et al., 2012; van den Heuvel et al., 2000). Immunological impacts were observed in caged rainbow trout exposed to those same waters for 21 d, including decreased total leukocytes, and reduced antibody production against *Aeromonas salmonicida* (McNeill et al., 2012). A laboratory exposure demonstrated only minor short-term effects on blood leukocytes using an intraperitoneal (I.P.) injection of NAs extracted from oil sands tailings waters (MacDonald et al., 2013).

The purpose of this research was to determine whether oils sands-influenced waters, and oil sands-derived NAs have the potential to be immunotoxic to rainbow trout in a laboratory exposure. The first hypothesis is that exposure to environmentally sourced water that contains oil sands-derived NAs will cause an immune suppression in the laboratory. The second hypothesis is that NAs, extracted from similar waters, will cause the same immunological effects to the pond waters. A method was established wherein

rainbow trout were exposed to pond water, waterborne extracted NAs, or waterborne BaP for 7 d. The main immune endpoints were the distribution of thrombocytes, B-lymphocytes, myeloid cells, and T-lymphocytes in blood, spleen, head kidney, and gill. Following exposures, a subset of trout were injected with formalin inactivated *A. salmonicida* and held in laboratory water for 21 d to measure antibody production. Water chemistry, NAs and BaP fluorescent bile metabolites as well as liver CYP1A induction were also determined after the 7 and 21 d exposure.

3.2 Methods

3.2.1 Experimental design

A total of three experiments were conducted. In the first experiment, rainbow trout were exposed to pond waters influenced by oil sands activities from the Fort McMurray region. In the second experiment, trout were exposed to NAs extracted and isolated from weather oil sands tailings water. In the third experiment, the effects of waterborne BaP, were examined.

The following experimental design was used for all three experiments and replicated in two separately timed trials for each experiment. This study was approved by the University of Prince Edward Island Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Rainbow trout (*Oncorhynchus mykiss*) were purchased from Ocean Trout Farms Ltd. (Brookvale, PE, Canada) and held in a circular 312 L flow through tank prior to exposures. Twenty-two fish were transferred from the holding tank to three 60 L flow-through, epoxy-coated, fibreglass rectangular tanks and were acclimated for one week prior to the start of

exposure. Fish were exposed to experimental waters for 7 d using a flow through system comprised of glass reservoirs and a peristaltic pump with a 90 % replacement time of 30 h. For all experiments, six fish from each tank were sacrificed for examination of leukocyte endpoints as well as fluorescent bile metabolites and hepatic 7-ethoxyresorufin-O-deethlyase (EROD) induction at 7 d. The remaining 16 fish from each tank were injected with *A. salmonicida* (10^8 particles/kg in PBS in 100 µL), or PBS alone using insulin needles ($29^{1/2}$G, 3/10cc, BD, Mississauga, Canada) and held in a 312 L flow through tank with laboratory water for an additional 21 d. Trout were then sacrificed and measured for similar endpoints as the 7 d exposure as well as antibody production against *A. salmonicida*. The mean weight of the rainbow trout used for the experiments was 59.4 ± 1.8 g. Mean dissolved oxygen saturation, temperature, salinity, and pH of the laboratory water was 98.1 %, 11.3 °C, 0.50 µS/cm, and 8.15, respectively.

3.2.1.1 Experiment 1: pond waters

Rainbow trout were exposed to either oil sands-influenced water, relatively uncontaminated water from the Athabasca oil sands region, or laboratory water. The oil sands-influenced water was derived from an experimental pond, Demonstration Pond, a 19-year old pond used to demonstrate water capping of oil sands tailings as a reclamation method. Demonstration Pond contains 70,000 m^3 of natural surface water (drainage water from muskeg area) that was pumped over 70,000 m^3 of mature oil sands fine tailings (van den Heuvel et al., 1999a). The relatively unimpacted water used for this experiment was obtained from Horizon Lake. Horizon Lake is an artificial lake constructed in 2008. This lake was built as a compensation for fish habitat loss and has now become a self-

sustaining ecosystem. A total of 2,000 L of exposure water was collected by pumping it into 1,000 L food-grade polyethylene totes. Totes were shipped by ground from Alberta to the University of Prince Edward Island and experiments were initiated within two weeks of receiving the waters. The third experimental water was laboratory well water. The mean temperature of the experimental waters was 15.2 ± 0.03 °C throughout the exposure.

3.2.1.2 Experiment 2: naphthenic acids

NAs for fish exposure were extracted by acid precipitation using aged (17 year old; Pond 10) oil sands tailings water using a modified extraction method from Frank et al. (2006) and characterization is described in detail by MacDonald et al. (2013). Briefly, water was acidified to pH 2 ± 0.2 with H_2SO_4 (Sigma, Oakville, Canada), precipitate removed and re-dissolved in 0.1 M NaOH (Sigma). Particulate matter was removed via centrifugation and humic material was removed via DEAE cellulose filtration. After liquid-liquid extraction with dichloromethane to remove neutrals, NAs were re-precipitated, washed with distilled water and freeze dried to produce a solid material. Classical NAs structures ($C_nH_{2n+z}O_2$) comprised 91 % of the extracted and isolated NAs (MacDonald et al., 2013). Stock solutions of NAs in 0.1 M NaOH were used to make up dilutions of 1 and 8 mg/L NAs in the glass reservoirs. The control for the NAs experiment was laboratory water spiked with NaOH carrier. The mean water temperature was 11.3 ± 0.02 °C throughout the NAs exposure.

3.2.1.3 Experiment 3: benzo[a]pyrene

The flow-through BaP exposure was conducted by pumping BaP (Sigma) dissolved in acetone carrier into a flow of laboratory water, both of which were delivered using peristaltic pumps with a 90 % replacement time of 30 h. Nominal concentrations were 50 μg/L and 200 μg/L of BaP. The control for this experiment consisted of acetone carrier only (0.002 %). The mean water temperature for the BaP exposure was 12.7 ± 0.03 °C throughout the exposure.

3.2.2 Sampling

Fish were anesthetized in 0.1 g/L tricaine methanesulfonate (MS-222; Argent, WA, USA), weighed, and fork length measured. Blood was removed (200 – 400 μL) from the dorsal artery using heparinized 23G needles (BD) and samples were placed in a 5 mL heparinized vacutainer (BD). A subsample of blood (5 μL) was removed and diluted in 1 mL of PBS/EDTA (Sigma) for total cell count. The remainder of blood was placed in 3 mL of L-15 media with glutamine (Sigma) for differential white cell count. For the 21 d *A. salmonicida* challenge only, a second blood sample (200 μL) was removed first using non-heparinized 23G needles (BD) and placed in a 1 mL non-heparinized microtainer SST (BD) containing silica and gel, initiating clotting and allowing for serum separation. Spleen, head kidney, and gill samples were removed and placed in 3 mL of L-15 media with glutamine (gills and spleen) or PBS/EDTA (head kidney) on ice. Liver samples were removed and placed in cryovials (Ultident Scientific, St. Laurent, Canada) and flash frozen in liquid nitrogen, and later stored at -80 °C. Bile samples were placed in microcentrifuge tubes (Ultident Scientific) and stored at -20 °C.

3.2.3 Total leukocyte count

Spleen, head kidney, and gill samples were homogenized using Tenbroek tissue grinders (Wheaton Science International, NJ, USA). The homogenate was then filtered through a 100 μm Nitex mesh and subsamples of 50 μL were diluted with 1 mL of PBS/EDTA. The remainder of the samples was used for differential leukocyte counts. The lipophilic dye, 3,3-dihexyloxacarbocyanine ($DiOC_6$) was added and incubated at room temperature for 15 min (Inoue et al., 2002) and a volume of 250 μL was transferred to FACS tubes (VWR, Mississauga, Canada). Multicolour CountBrite counting beads (Invitrogen, Burlington, Canada) (20 μL) containing 20,000 multicolour beads were added to the FACS tubes and read on a BD FACSCalibur flow cytometer equipped with 488 nm and 635 nm lasers. The samples were read until 1,000 beads were passed through the fluorescent channel 4 (FL4, red). Erythrocytes and leukocytes were separated and absolute counts were determined using a plot of side scatter vs. fluorescent channel (FL1, green).

3.2.4 Differential leukocyte count

Leukocytes were isolated by density gradient (Secombes, 1990). Blood and homogenized samples were carefully layered over 3 mL of Lympholyte-H (1.0770 g/cm^3; Cedarlane Laboratories, Burlington, Canada) and centrifuged at 600 g for 40 min at 4 °C. The leukocytes found at the interface were removed and washed with 5 mL of PBS/EDTA and cells were re-suspended in L-15 media. Cells were counted using a hemocytometer in order to obtain 400,000 cells per well in 96 round bottom plates.

Monoclonal antibodies used for the differential leukocyte staining have been previously described by MacDonald et al. (2013). B-lymphocytes and thrombocytes are stained using directly labeled antibodies while myeloid and T-lymphocytes are stained using conjugated monoclonal antibodies. Plates were incubated with their appropriate stains for 1 h at 4 °C and spun at 250 g for 4 min at 4 °C and re-suspended cells were washed with 100 μL of PBS/EDTA. For those antibodies requiring a secondary antibody, washing was not conducted until after the secondary antibody had incubated for 1 h at 4 °C, after which the procedure was identical to that of labeled primary antibodies. The cell suspensions were counted on a BD FACSCalibur to a total count of 5,000 events, each representing one cell. The forward scatter vs. side scatter plot was gated to remove debris and dead cells and erythrocytes were gated out on a plot of FL1 vs. FL3. Stained cells were evaluated using the appropriate fluorescence vs. forward scatter for each antibody in comparison to unstained cells.

3.2.5 Fluorescent bile metabolites

Bile samples were analyzed after diluting 100-fold in high-performance liquid chromatography (HPLC) grade water (Caledon Laboratories, Georgetown, Canada). Samples were filtered into glass autosampler vials using 13 mm polypropylene syringe filters with a 0.45 μm pore size (Pall, St. Laurent, Canada). A Varian Prostar model 240 HPLC pump, a model 410 autosampler, and model 363 fluorescence detectors were used for quantification of fluorescent metabolites. Separations were conducted with a 150 × 4.6 mm Varian Microsob-MV C18 column at a flow rate of 1 mL/min at 35 °C. Solvent elution profile started at 5 % acetonitrile (Caledon) and 95 % HPLC grade water. These

ratios were changed to 98 % acetonitrile and 2 % water gradually over a 25 min period with this ratio being held until the end of the run (5 min). Excitation and emission wavelengths used were that of phenanthrene (256 nm and 380 nm, respectively) and BaP (380 nm and 430 nm, respectively). The total peak area between 3 and 15 min was summed and this was compared to the standard curve for phenanthrene and BaP to derive equivalent concentrations in μg/mL of bile.

3.2.6 Hepatic 7-ethoxyresorufin-O-deethlyase (EROD)

Hepatic 7-ethoxyresorufin-O-deethlyase (EROD) activity as a measure of liver cytochrome P4501A (CYP1A) enzyme activity was estimated in post-mitochondrial supernatant (PMS) using methods previously described by van den Heuvel et al. (1995a). Liver samples were homogenized (0.1 M phosphate, 1 mM EDTA, 1 mM dithiothreitol, and 20 % glycerol, pH 7.4) using a glass/Teflon homogenizer and centrifuged at 9,000 g to obtain the PMS. EROD reaction mixture contained 0.1 M HEPES buffer pH 7.8 (Sigma), 5.0 mM Mg^{2+}, 0.5 mM NADPH (Sigma), 1.5 M 7-ethoxyresorufin (Sigma) and about 0.5 mg/mL of PMS protein. EROD activity was determined in 96 well black plates and after 10 min the reaction was terminated with acetonitrile and read on a fluorescence plate reader (Bio-Tek FLx800) with 528 nm excitation and 590 nm emission filters. Protein content was estimated from fluorescamine fluorescence (390 nm excitation, 460 nm emission filters) against bovine serum albumin standard (Sigma).

3.2.7 A. salmonicida antibody ELISA

Enzyme-linked immunosorbent assays (ELISAs) were used to determine the relative quantity of inactivated *A. salmonicida* antibody found in trout sera (Köllner and Kotterba, 2002). Inactivated *A. salmonicida* (6.4×10^7 particles/mL, 200 µL/well) was added to 96 well flat-bottom microtest ELISA plates (BD) and incubated overnight at 4 °C. Protein free blocking buffer with 1 % ovalbumin (Pierce, Rockford, IL, USA) was added to the plates and incubated for 1 h at room temperature. The plates were then washed three times (PBS, pH 7.4, 0.05 % Tween, Sigma). Trout serum was diluted 1:10 in PBS washing buffer and added to the plates and an 8-point antibody titer was conducted (serial two-fold dilutions). Plates were incubated for 1 h at 4 °C and washed three times with PBS washing buffer. The monoclonal mouse–anti trout IgM antibody (mab 4c10) (1:100 dilution in PBS tween) was added to each well and was incubated for 1 h at 4 °C. The antibody was discharged and the plates were washed three times with PBS washing buffer. The conjugate goat anti-mouse-IgG-HRP-peroxidase conjugate (Pierce, Germany) (1:1250 dilution in PBS tween) was added to the plates and incubated for 1 h at 4 °C. The conjugate was discharged and washed with PBS washing buffer three times. The SigmaFAST OPD substrate (Sigma) was added to the plates and incubated for 30 min at room temperature. Adding 100 µL of 3 M HCl in each well terminated the reaction, which was then read at 490 nm by a micro-plate reader. The antibody titer was determined as being the first serum concentration with an absorbance greater than three times the mean blank value. This was then expressed as the negative logarithm of the antibody titer.

3.2.8 Water chemistry

For pond waters, total NAs in water using a Fourier transform infrared spectroscopy analysis following dichloromethane extraction of water were provided by industry partners (Jivraj et al., 1995). The exposure waters were evaluated by liquid chromatography-high resolution mass spectrometry (LC-HRMS) using both a C18 extraction, and a direct injection of filtered exposure water to determine the NAs concentration. The pond water and extracted NAs water samples were acidified to pH 2 with concentrated H_2SO_4. For the C18 method, subsamples of 50 mL were passed through a C18/18 cartridge (500 mL; Scientific Products and Equipment, North York, ON, Canada). Cartridges were eluted with 10 mL of 1:1 MeOH/10 mM NH_4OH and 400 µL subsamples were added to autosampler vials with 10 µL of internal standard of 5 µg/mL stearic acid and myristic acid. For the direct injection method, acidified exposure water was filtered through a 0.45 µm syringe filter, and internal standards added. A Thermo Scientific Acella LC system interfaced with a Thermo Velos Orbitrap mass spectrometer equipped through an electrospray ionization interface was used to measure NAs. Samples were separated on an Agilent Zorbax extend C18 column (2.1 × 100 mm, 3.5 µm particle size) using a solvent profile starting with 50 % water/0.1 M formic acid and 50 % methanol/0.1 M formic acid held for the first minute and increasing to 100 % methanol/0.1 M formic acid by 5 min and held for an additional 3 min. The mass spectrometer scan was from m/z 110 – 400 at 30,000 mass resolution. NAs concentration was evaluated against a standard solution of the extracted and purified NAs using seven ions (< 5 ppm from nominal m/z) that were observed to be highly specific to the mixture of NAs. According to the NAs formula $C_nH_{2n+z}O_2$, those ions (m/z) corresponded to C13

Z=-4 (209.1536), C13 Z=-2 (211.1693), C15 Z=-8 (233.1536), C15 Z=-6 (235.1693), C15 Z=-4 (237.1849), C15 Z=-2 (239.2006), C17 Z=-8 (261.1849). All seven ions were used to quantify total NAs against the extracted NAs standard and the individual results for each of the ions were averaged to give a total NAs concentration. Due to poor response of the higher molecular weight ions, only m/z 209.1536, 211.1693, and 233.1536 ions were used in the quantification of the directly measured (unextracted) water samples.

Acid extractable structures not corresponding to the $C_nH_{2n+z}O_2$ formula are known to be present in weathered oil sands material, particularly O_3 and O_4 NAs, presumed to be alcohols of the primary NA structures. The profile of all O_2, O_3, and O_4 NAs were evaluated on C18 extracted waters, or NAs solutions by direct infusion into the mass spectrometer using a Surveyor syringe pump at 2.5 µL/min. Ratios of O_2, O_3, and O_4 NAs were derived by summing the total ion intensity of all ions within those three groups corresponding to m/z ratios of possible structures from 5 to 30 carbons and from Z=0 to Z=-30.

3.2.9 Statistics

Trout weight, fork length, liver and spleen size were analyzed using analysis of covariance (ANCOVA) using logarithmically transformed values. Somatic data were expressed as indices for presentation purposes using the least squares means and covariate means from the ANCOVA. Condition factor was calculated as weight ÷ length^3 × 100. Liver and spleen somatic indices were calculated as organ weight ÷ body weigh × 100. All remaining results were analyzed by testing for normality and homogeneity of

variance (Levene's and Brown-Forsythe tests) with appropriate transformations where those assumptions were not met. Total leukocyte counts in blood and tissue were used in conjunction with differential leukocyte counts to derive absolute leukocyte counts for each specific cell type in each tissue. A full factorial two-way ANOVA comparing experimental trial and dose effect was performed, followed by a post-hoc test of treatments against controls using Dunnett's test. STATISTICA version 8.0 was used for all statistics using an experiment-wise alpha of 0.05.

3.3 Results

3.3.1 Water chemistry

LC-HRMS analysis demonstrated similar values between both methods of measuring NAs in exposure waters: C18 extracts and direct injections of water samples (Table 3.1). In comparison, FTIR NAs determination methods report approximately two-fold higher values. The measured values for the 1 and 8 mg/L NAs concentrations were slightly lower than the nominal values. In addition to the standard $C_nH_{2n+z}O_2$ NAs structure, substantial amounts of NAs with additional oxygen, presumed to be NAs alcohols can be observed in weathered oil sands-influenced waters. In Demonstration Pond, O_3 and O_4 NAs comprised more than 10 % of the mixture. For comparison purposes, these compounds were more than 40 % of the NAs in Pond 10, the water from which the purified NAs were extracted. This demonstrates that the acid precipitation extraction process of NAs eliminated O_3 and O_4 compounds as the purified NAs were virtually all O_2 compounds. Demonstration Pond had higher pH and salinity when compared to Horizon Lake and laboratory water controls.

Table 3.1 Water chemistry parameters (n, S.E.M.) for the 7 d pond water and NAs exposure. NAs percentages demonstrate the relative concentration of each compound family, the NAs O_2, NAs with one additional oxygen (O_3), and with two additional oxygens (O_4) (ion charge of -1) based on parent ion intensity.

Experiment	**pH**	**Conductivity (µS/cm)**	**NA mg/L (FTIR)**	**NA mg/L (C18 extract)**	**NA mg/L (direct injection)**	**NA (%)**		
						O_2	**O_3**	**O_4**
Control	8.32 (4, 0.07)	811.0 (4, 0.003)		0.16	0.07 (2, 0.22)			
Horizon Lake	8.20 (4, 0.06)	428.0 (4, 0.01)	< 1	0.27	0.64 (2, 0.05)	26.8	23.7	49.6
Demonstration Pond	8.94 (4, 0.11)	1881.8 (4, 0.02)	13.0	5.77	6.25 (2, 0.19)	88.7	9.3	2.0
Control	8.33 (4, 0.03)	988.0 (4, 0.01)		0.18	0.34 (2, 0.55)			
1 mg/L NA	8.31 (4, 0.04)	984.7 (4, 0.01)		0.73	0.76 (2, 0.02)	99.8	0.15	0.03
8 mg/L NA	8.26 (4, 0.05)	988.3 (4, 0.01)		6.04	6.62 (2, 0.05)	99.8	0.15	0.03
Pond 10[a]						60.0	22.9	17.2

[a] Pond 10 was used for the extraction of NAs used for the exposures.

3.3.2 Experiment 1: pond waters

There was a single mortality observed in the group exposed to Demonstration Pond water for 7 d. There were no differences in weight as it covaries with length (condition factor) or liver size as it covaries with body weight (presented as LSI) between pond water exposure groups after 7 d (Table 3.2). However, there was a statistically significant 30 % decrease in spleen size as it covaries with body weight (presented as SSI) in Demonstration Pond when compared to the control group (Table 3.2). There were no significant differences in blood total erythrocyte counts.

Fish exposed to Demonstration Pond water showed a significant decrease in B-lymphocytes (48 %) and T-lymphocytes (55 %) in blood (Fig. 3.1). Significant decreases of B-lymphocytes by 83 % and myeloid cells by 46 % in spleen were also observed after 7 d of exposure (Fig. 3.2). Demonstration Pond water-exposed fish also showed an increase in B-lymphocytes by 57 % in head kidney after 7 d (Fig. 3.3). There were no differences in leukocytes observed in gill tissue (Fig. 3.4). Differential cell counts were also measured at 21 d post *A. salmonicida* injection and demonstrated no change in cell type population between groups (data not shown). However, there was a decrease in antibody production against *A. salmonicida* in Horizon Lake by 36 % and in Demonstration Pond by 49 % (Fig. 3.5). There were no statistical significant differences in hepatic CYP1A activity after 7 d (Fig. 3.6). There was an increase in fluorescent bile metabolites at phenanthrene wavelengths in both Demonstration Pond and Horizon Lake and an increase at BaP wavelengths in Demonstration Pond after 7 d (Fig. 3.7).

Table 3.2 Condition factor, liver and spleen somatic index and erythrocytes in blood (n, S.E.) in rainbow trout after 7 d exposure. Asterisks demonstrate a statistically significant difference between the exposed group and the control by two-way ANOVA analysis.

Experiment	**Condition Factor**	**Liver Somatic Index**	**Spleen Somatic Index**	**Blood erythrocytes (billion cells mL^{-1})**
Control	0.99 (12, 0.01)	0.92 (12, 0.01)	0.13 (12, 0.05)	1.23 (12, 0.06)
Horizon Lake	1.00 (11, 0.01)	0.88 (12, 0.01)	0.11 (12, 0.05)	1.38 (12, 0.09)
Demonstration Pond	1.01 (12, 0.01)	1.02 (12, 0.01)	0.09 (12, 0.05)*	1.27 (12, 0.10)
Control	1.07 (12, 0.01)	1.06 (12, 0.01)	0.17 (12, 0.05)	1.42 (12, 0.06)
1 mg/L NA	1.05 (12, 0.01)	1.16 (12, 0.20)*	0.20 (12, 0.05)	1.33 (12, 0.05)
8 mg/L NA	1.03 (12, 0.01)	1.43 (12, 0.02)*	0.18 (12, 0.05)	1.30 (12, 0.05)
Control	1.06 (11, 0.01)	1.02 (11, 0.02)	0.11 (11, 0.06)	1.39 (12, 0.10)
50 μg/L BaP	1.02 (12, 0.01)	1.13 (12, 0.02)	0.12 (12, 0.06)	1.38 (12, 0.07)
200 μg/L BaP	1.01 (12, 0.01)	1.20 (12, 0.02)*	0.08 (12, 0.06)	1.67 (12, 0.07)*

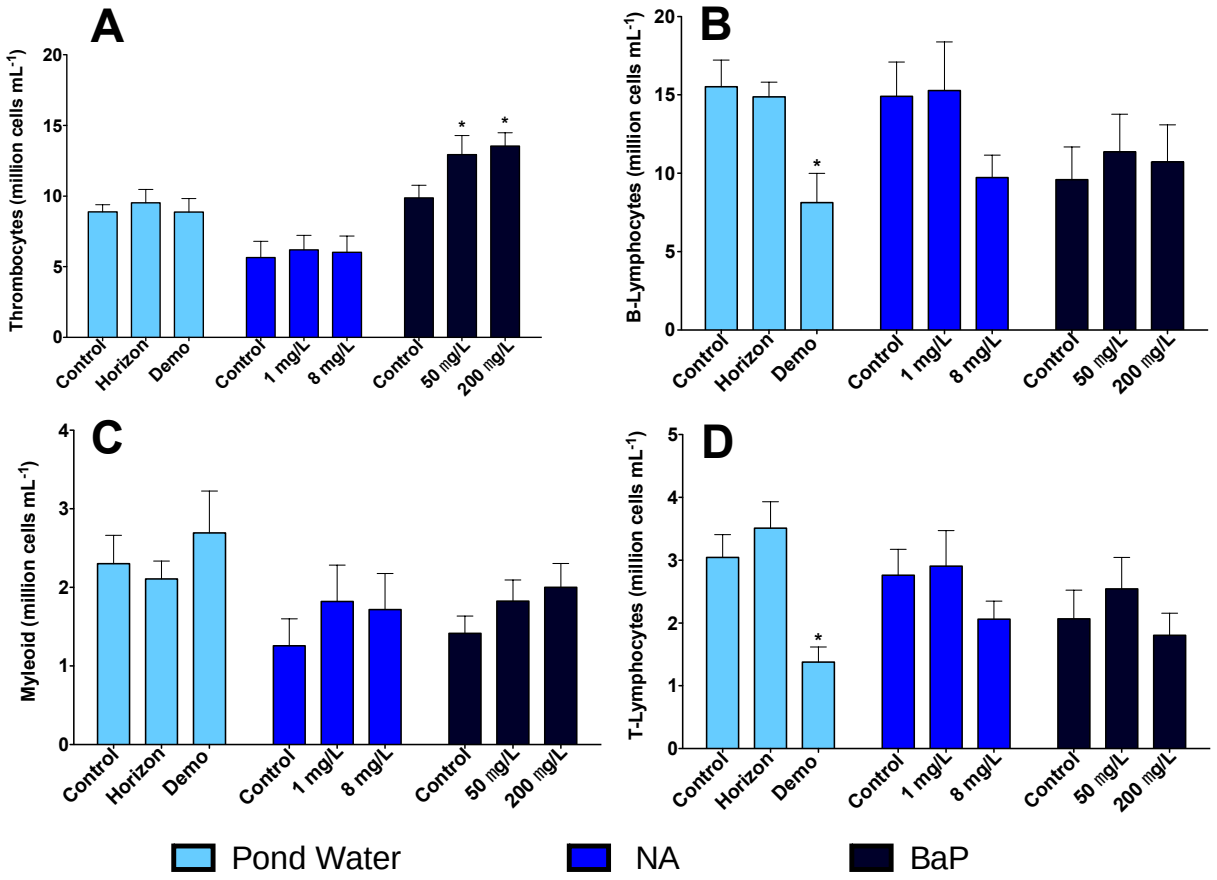

Figure 3.1 Mean blood total thrombocyte (A), B-lymphocyte (B), myeloid (C), T-lymphocyte (D) counts after 7 d exposure to pond water, NAs and BaP. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=12/group). Asterisk indicates a significant difference between the exposed and control group within each experiment by two-way ANOVA with Dunnett's test.

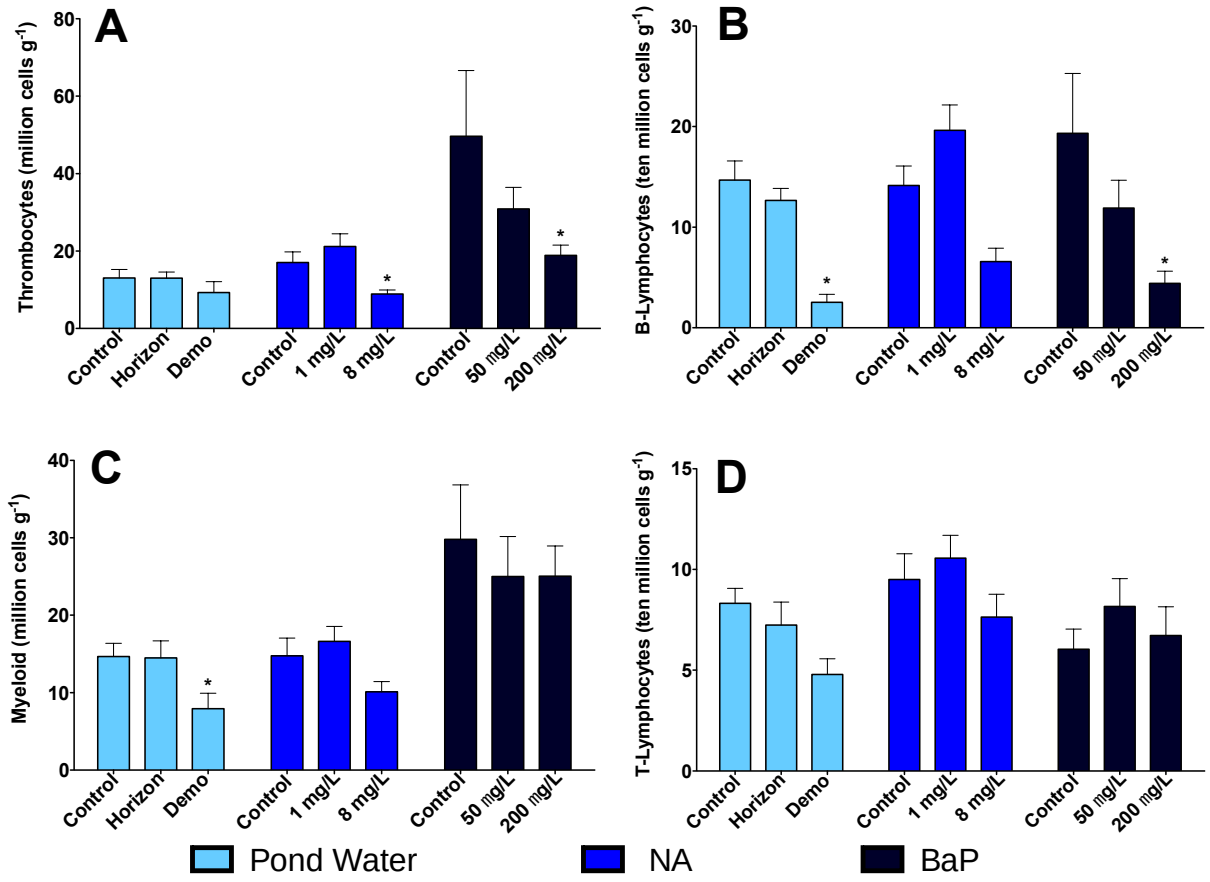

Figure 3.2 Mean spleen total thrombocyte (A), B-lymphocyte (B), myeloid (C), T-lymphocyte (D) count after 7 d exposure to pond water, NAs and BaP. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=12/group). Asterisk indicates a significant difference between the exposed and control group within each experiment by two-way ANOVA with Dunnett's test.

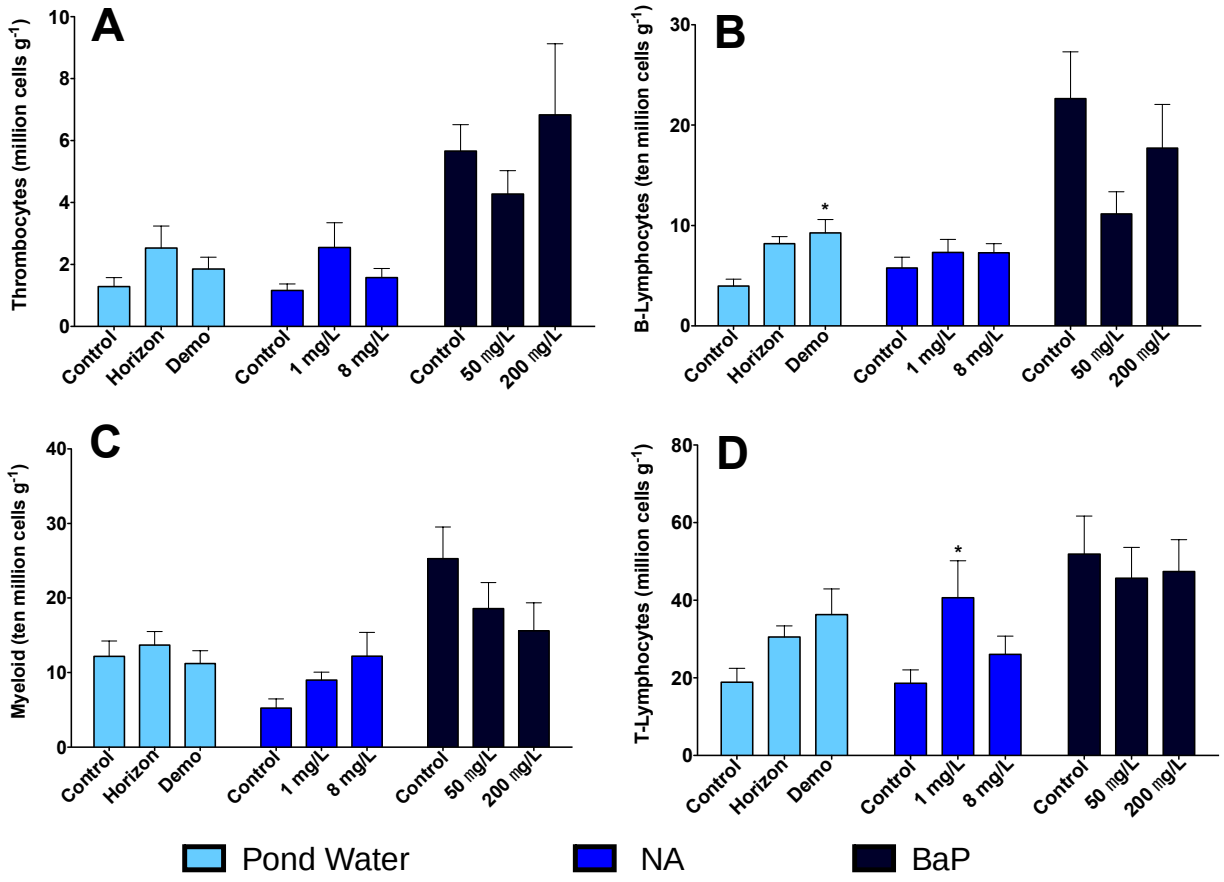

Figure 3.3 Mean head kidney total thrombocyte (A), B-lymphocyte (B), myeloid (C), T-lymphocyte (D) count after 7 d exposure to pond water, NAs and BaP. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=12/group). Asterisk indicates a significant difference between the exposed and control group within each experiment by two-way ANOVA with Dunnett's test.

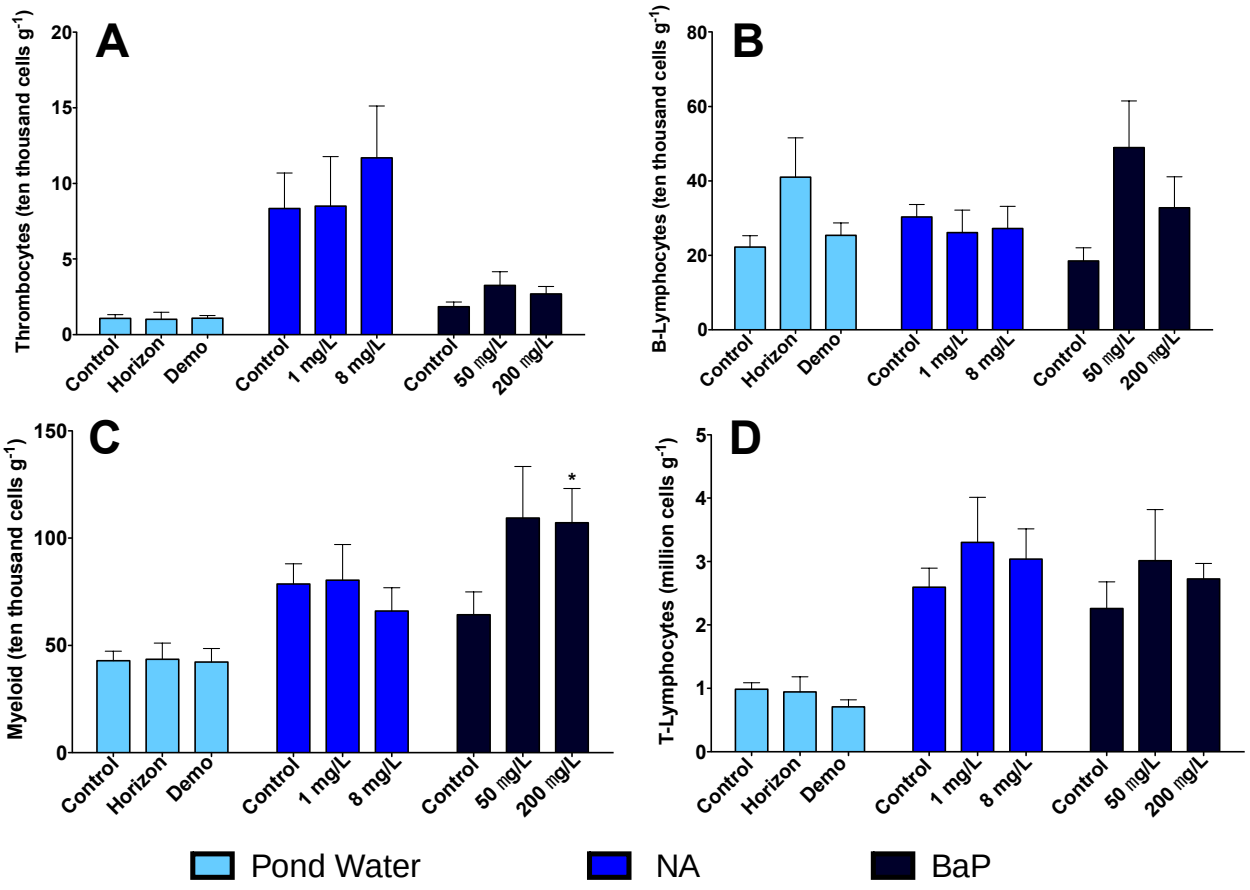

Figure 3.4 Mean gill total thrombocyte (A), B-lymphocyte (B), myeloid (C), T-lymphocyte (D) count after 7 d exposure to pond water, NAs and BaP. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=12/group). Asterisk indicates a significant difference between the exposed and control group within each experiment by two-way ANOVA with Dunnett's test.

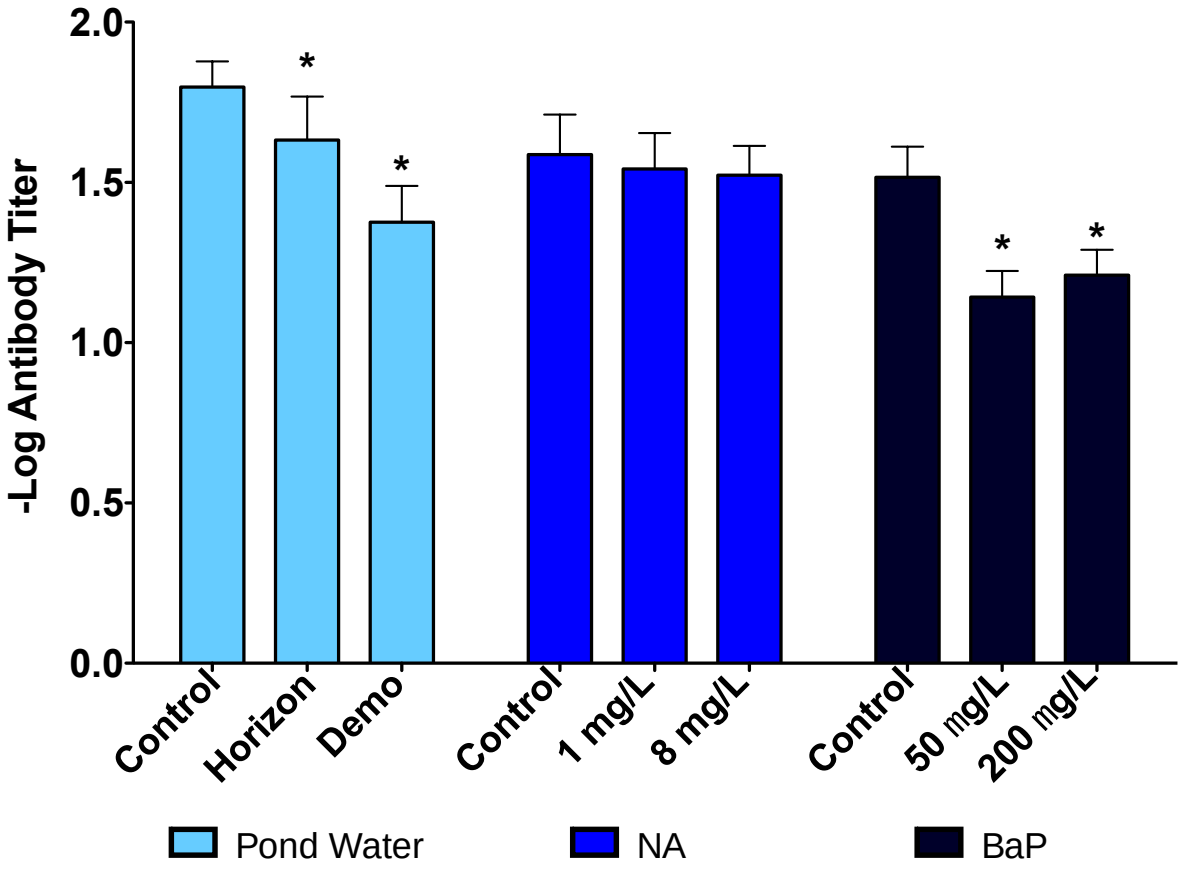

Figure 3.5 Mean *A. salmonicida* antibody titer for rainbow trout injected with *A. salmonicida* after 7 d exposure to pond water, NAs, BaP and subsequently held in clean water for 21 d. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=20/group). Asterisks indicate significant differences between the exposed groups to the control by two-way ANOVA followed by Dunnett's test.

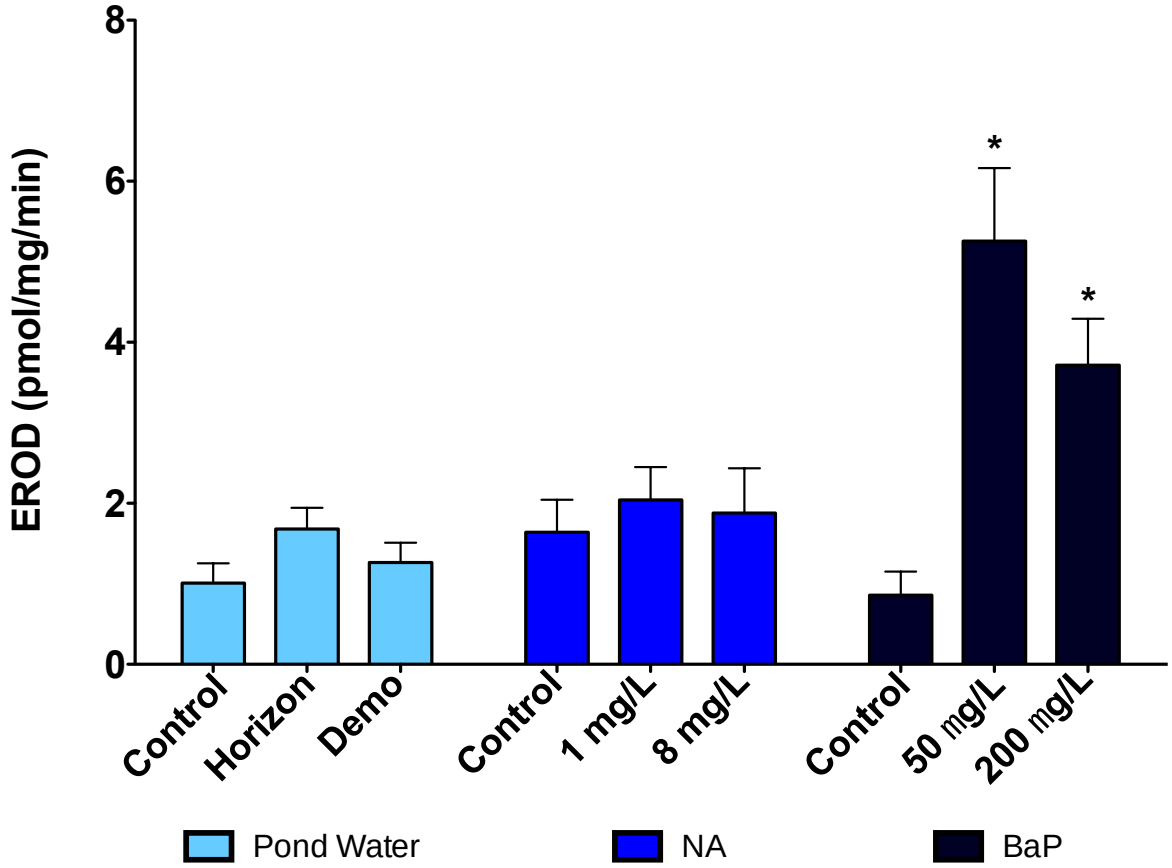

Figure 3.6 Mean hepatic 7-ethoxyresorufin-O-deethlase activity after 7 d exposure to pond water, NAs and BaP. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=18/group). Asterisks indicate significant difference between the exposed groups to the control by two-way ANOVA followed by Dunnett's test.

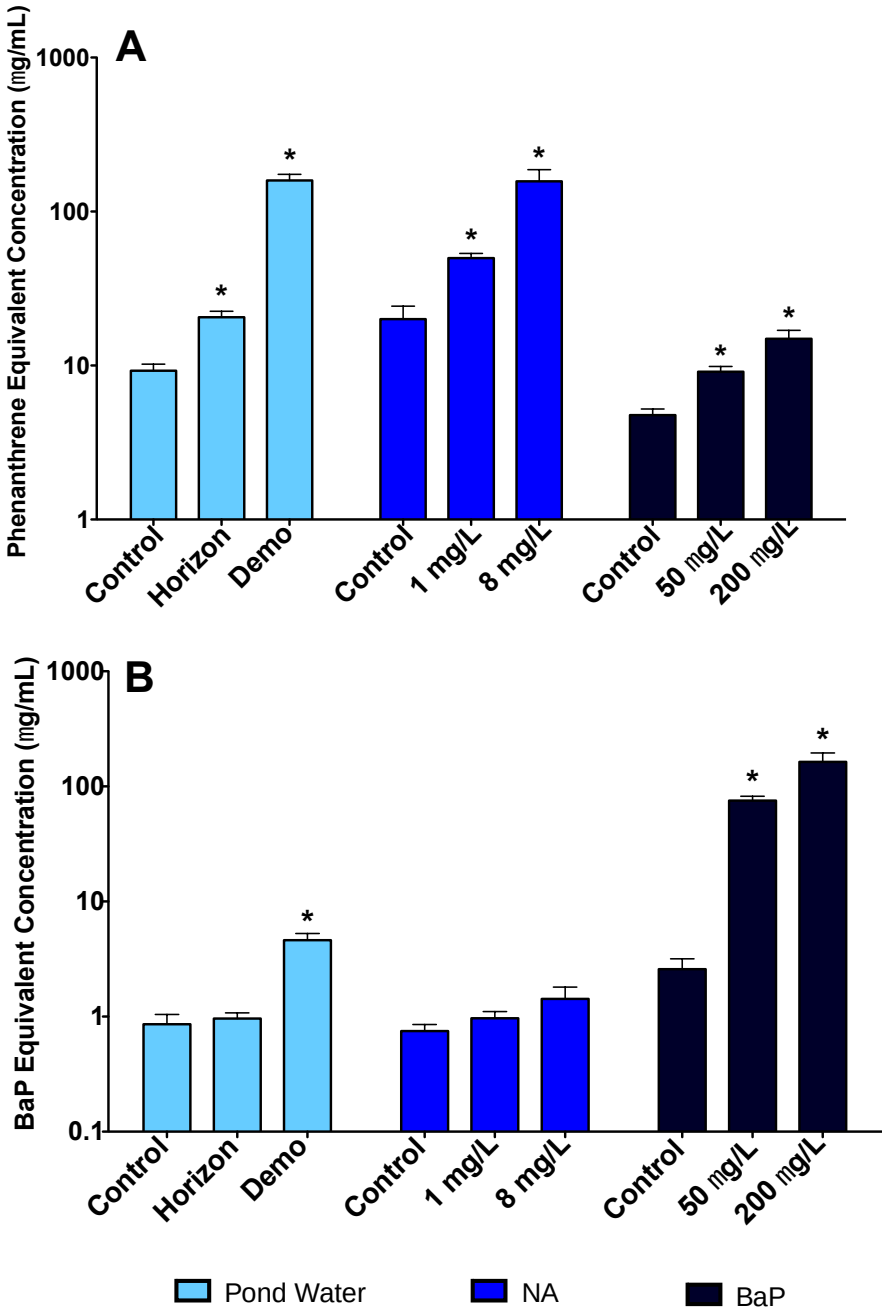

Figure 3.7 Mean biliary phenanthrene (A) and BaP (B) equivalent concentration determined by HPLC-fluorescence after 7 d exposure to pond water, NAs and BaP. Different colour bars indicate the three separate experiments. Average and S.E.M. are shown (n=18/group). Asterisks indicate significant difference between the exposed groups to the control by two-way ANOVA followed by Dunnett's test.

3.3.3 Experiment 2: naphthenic acids

The waterborne extracted NAs exposure had a single mortality in each of the following groups: control + PBS injected group, 1 mg/L NAs + PBS injected group and 8 mg/L NAs + *A. salmonicida* injected group. There were no significant differences in weight as it covaries with length or in spleen size corrected for body weight (Table 3.2). There was a significant increase in liver size at both the 1 and 8 mg/L extracted NAs doses (Table 3.2). There were no changes in blood erythrocyte counts.

There was a significant decrease in thrombocytes in spleen by 47 % at 8 mg/L (Fig. 3.2) and an increase in T-lymphocytes by 54 % at 1 mg/L in head kidney after 7 d exposure (Fig. 3.3). After the 21 d exposure, the differential leukocyte cell counts demonstrated no differences amongst groups (data not shown). There were no significant changes in antibody production against *A. salmonicida* after 21 d (Fig. 3.5). There were no statistically significant differences in hepatic CYP1A induction in liver with exposure to extracted NAs after 7 d (Fig. 3.6). There was a dose-dependent increase in bile metabolites measured at phenanthrene wavelength at both low and high dose of extracted NAs (Fig. 3.7A). However, there were no changes in bile metabolites measured at BaP wavelengths (Fig. 3.7B).

3.3.4 Experiment 3: benzo[a]pyrene

There was a single mortality in the 50 μg/L BaP + *A. salmonicida* injected group and a single mortality in the 200 μg/L BaP + PBS injected group. There was a significant increase in liver size corrected for body weight at 200 μg/L BaP as compared to the control group and a significant increase in blood erythrocyte concentrations at 200 μg/L

(Table 3.2). There were no significant differences in weight as it covaries with length or in spleen size corrected for body weight (Table 3.2).

There was a significant increase in thrombocytes in blood in both 50 μg/L (23 %) and 200 μg/L (27 %) BaP groups (Fig. 3.1) and an increase in myeloid cells in gills (39 %) at 200 μg/L (Fig. 3.4). B-lymphocytes and thrombocytes decreased at 200 μg/L BaP after 7 d in spleen by 77 % and 63 %, respectively (Fig. 3.2). There were no significant differences in differential leukocytes in any tissue after 21 d exposure (results not shown). This experiment demonstrated a significant decrease in *A. salmonicida* circulating antibodies in both low and high doses of BaP (Fig. 3.5). There was a significant increase in CYP1A induction in liver with both low and high doses of BaP after 7 d (Fig. 3.6). There was a significant increase in bile metabolites measured at both phenanthrene and BaP wavelength with both low and high dose of BaP (Fig. 3.7), although BaP equivalent concentration increased more than 100-fold, while phenanthrene equivalent concentration increased by less than 10-fold.

3.4 Discussion

Rainbow trout exposed to oil sands-influenced waters exhibited decreased leukocytes in blood, spleen and head kidney as well as a decrease in antibody production against *A. salmonicida*. However, NAs derived from oil sands-influenced waters, at similar concentrations to those found in the surface waters tested, caused no comparable effects. A positive control exposure showed a suite of effects that were similar but not identical to those found with oil sands-influenced waters. Trout exposed to oil sands-influenced waters and extracted NAs demonstrated elevated levels of fluorescent

metabolites in bile. However, hepatic CYP1A as indicated by EROD activity was not increased by either oil sands-influenced waters or by extracted NAs.

The immunotoxic responses observed following exposure to oil sands-influenced water sourced from Demonstration Pond were consistent with previous responses reported in yellow perch and rainbow trout inhabiting Demonstration Pond. In a series of studies conducted since 1995, yellow perch were released into Demonstration Pond and after three months exhibited two disease pathologies, fin erosion and skin lesions (Hogan et al., 2011; van den Heuvel et al., 2000). Skin lesions were identified as lymphocystis disease, and the causative agent, lymphocystis disease virus, can be detected in asymptomatic individuals in a Northern Alberta reference population (Palmer et al., 2012). Thus, it appeared that exposure to Demonstration Pond water caused changes that allowed the virus to overcome the perch immune system. An *in situ* trout experiment conducted by McNeil et al. (2012) caged rainbow trout in Demonstration Pond for 21 d. This exposure caused some initial mortality, a decrease in total leukocytes in blood, reduced production of antibodies to *A. salmonicida,* and decreased spleen size. Unlike the experiment at hand, facilities were not available in the field to examine specific leukocyte populations in blood or tissue, though blood smears indicated the lymphocytes were depressed in blood. The exposure protocol employed here was able to show results consistent with the field experiment with only 7 d exposure to Demonstration Pond water.

Unlike Demonstration Pond water, oil sands-derived NAs did not possess significant immunotoxic potential. This research follows from a previous study that used I.P. injection of the same source of extracted NAs used herein (MacDonald et al., 2013). In this previous study, there were no significant decreases observed on blood or tissue

leukocytes at 21 d post injection, though some stimulation of leukocytes in blood was observed and attributed to a mild inflammatory response. The only other immunotoxicity studies with fish have been conducted with commercial sources of NAs. Goldfish exposed to waterborne commercial NAs exhibited initial stimulation of pro-inflammatory gene expression in gills, kidney and spleen (Hagen et al., 2012). Another study reported that exposure of mouse bone marrow cells to a dichloromethane extract of oil sands-influenced waters and commercial NAs resulted in a reduction in phagocytosis associated with alteration in pro-inflammatory cytokines (Garcia-Garcia et al., 2011). However, commercial NAs mixtures bear little resemblance to the chemical profile found in aged oil-sands waters making study comparisons impossible (MacDonald et al., 2013). It has also been suggested that commercial mixtures occasionally contain toxic components other than carboxylic acids such as $C_{0–6}$ alkylphenols (West et al., 2011) that could change the toxicity of the mixture. These 'aromatic' acids can also be found in oil sands-influenced water (Jones et al., 2012). While the NAs used here did not cause immunotoxicity, this does not imply that carboxylic acids, or acid extractable compounds are not responsible for immune toxicity of Demonstration Pond water as the extraction and purification procedure used was highly selective for isolating structures conforming to the $C_nH_{2n+z}O_2$ formula thereby removing many other organic structures.

The nature of the agent in Demonstration Pond water that is causative of immune impacts remains uncertain. It has been recently observed that increasing pH could be an issue for fish health in systems employing a water-capped tailings approach as carbonates seep into the surface water as tailings consolidate, raising pH (van den Heuvel et al., 2012). Although Demonstration Pond has a relatively high pH of 8.94 in this study, and

up to 9.5 in field measurements (van den Heuvel et al., 2012), investigations have shown that rainbow trout are able to cope with acute high pH (pH > 9.0) exposure through their ammonia excretion ability, acid-base homeostasis and electrolyte balance (Wilkie et al., 1996). An increase in disease was also observed in the mid-1990's in yellow perch released into another experimental system (South Bison Pond) that was primarily influenced by unextracted oil sands material, and high pH was not observed in this pond water (van den Heuvel et al., 2000). Similarly, salinity in general, while elevated over natural surface waters is not thought to be responsible for causative immune effects. Recent studies using the South Bison Pond, with identical salinity to the Demonstration Pond, shows much less pronounced immune effects in yellow perch and trout in the South Bison Pond (Hogan et al., 2011; McNeill et al., 2012). The high levels of salt may in fact change the surfactant properties of the NAs in oil sands process-affected waters and reduce apparent toxicity (Samson et al., 2013).

Metals are not suspected as the immunotoxic agents in the Demonstration Pond water. Metal contamination, likely associated with deposition from atmospheric sources has been suggested as being a concern for the region (Kelly et al., 2010). However, despite being in the depositional epicenter, the Demonstration Pond has not shown evidence of influence from priority metals (van den Heuvel et al., 1999a; 2012). However, metals such as B, Sr and Li that are associated with marine clays, thus expected to have been derived from tailings, are elevated in Demonstration Pond water (van den Heuvel et al., 2012). Given the limited knowledge of the toxicity of these compounds, conclusions cannot be made on potential for immunotoxicity.

From our results, it appears that immunotoxic activity was not associated with

activation of CYP1A. Previous studies have consistently shown elevated CYP1A activity in yellow perch (van den Heuvel et al., 1999b), rainbow trout (McNeill et al., 2012), and white sucker (Arens et al., 2013) exposed to Demonstration Pond water. This is the first study to show the absence of CYP1A induction with this water source. There are a number of possible reasons for this including the possibility that induction in the pond environment was due to a dietary exposure to PAHs, which is supported by measured levels of PAHs in pond invertebrates (Wayland et al., 2008). However, it is also possible that the causative agents of CYP1A induction were labile and did not persist, or were removed through the shipping and storage of the pond waters. Regardless, this does indicate that immunotoxicity can proceed in the absence of CYP1A induction, suggesting that immunotoxicity is likely not Ah receptor-dependent. PAHs have been suspected of being immunotoxic agents in these pond waters due to the frequent observation of elevated CYP1A activity. The absence of CYP1A activity in the pond water experiments suggests that trout were not exposed to PAHs in these experiments, which in turn raises the likelihood that compounds other than PAHs may be involved in immunotoxicity in these experiments. Previously, bile fluorescence was also considered indicative of PAH exposure, however, it is known from the exposure to the purified NAs that this also is not the case.

Fluorescent bile metabolites measured by HPLC analysis at phenanthrene wavelengths were increased with exposure to oil sands-influenced waters and NAs exposure. Elevated phenanthrene equivalent concentration has been observed in yellow perch exposed to Demonstration Pond water (van den Heuvel et al., 1999b) and rainbow trout caged in this water (McNeil et al., 2012). A key difference between pond water and

NAs exposure in the experiments described here was the increased complexity/aromaticity of the pond water as indicated by elevated bile fluorescence at BaP wavelengths. This is likely because some of the more hydrophobic or neutral structures are removed from the extracted NAs though a dichloromethane extraction step in the purification (MacDonald et al., 2013). NAs possess weak fluorescence at wavelengths similar to the phenanthrene wavelengths used herein (Kavanagh et al., 2009; Mohamed et al., 2008). While those fish exposed to waterborne purified NAs showed elevated bile phenanthrene equivalent concentration, this result is contrary to a previous study using I.P. injection of the same material where no increase in bile fluorescence was observed. This suggests that NAs injected I.P. are not as available to the fish as with waterborne exposure used here. While bile fluorescence increased upon exposure to oil sands-influenced waters, it is not specifically indicative of the nature of the compounds to which fish are exposed in the case of oil sands exposures. Use of more specific techniques such as NAs analysis in bile could be employed to better understand the source of the bile metabolites.

The immunotoxicity of Demonstration Pond water bears some similarities to the effects characteristic of BaP, a known immunotoxic PAH. BaP was not only used as a positive control because it is a known immunotoxic effects on B-lymphocytes and T-lymphocytes (Logan et al., 2007), but also to compare the tissue specific pattern of that immunotoxicity to the pond water exposures. The most consistent and profound effect of BaP and pond water exposure was the depletion of B-lymphocytes from spleen. This has been repeatedly observed with I.P. injection of BaP in rainbow (MacDonald et al., 2013; Phalen et al., 2013). As the spleen is a storage organ for B-lymphocytes, it is likely that

these stores are depleted faster than they can be replaced when significant B-lymphocyte toxicity is occurring. While this response is similar in nature between pond water and BaP exposure, the lack of CYP1A activity previously mentioned makes it unlikely that PAHs are causative agents in the pond water exposure. Other responses were also dissimilar, notably the observations that BaP stimulated both erythrocyte and thrombocyte counts in blood and caused elevations in liver size, neither of which was observed with pond water.

While there is uncertainty about causative agents, the body of evidence suggests that organic compounds associated with oil sands are still the most likely immunotoxic agents. Yellow perch disease studies were conducted over a 17 year time period during which considerable changes in pond chemistry took place (van den Heuvel et al., 2012). During this period, perch disease incidence decreased in South Bison Pond, and increased in Demonstration Pond. The strongest correlate to this effect was the total NAs concentration as derived by FTIR methods (Hogan et al., 2011). While the NAs mixture tested here was not immunotoxic, this is only a refined subset of the complex suite of compounds in the mixture. Extraction procedures were selective for compounds that precipitate with acidification, and the process further selectively removed any neutral, or more hydrophobic compounds from the NAs mixture though dichloromethane extraction of the basic NAs solution. This dichloromethane extraction fraction induced CYP1A activity after 24 h using H4IIE-*luc* cells (Chap. 2). This fraction may include neutral compounds known to be present in the pond environment such as alkylated dibenzothiophenes (Wayland et al., 2008).

The mixture of carboxylic acids in oil sands-influenced waters is proving to be far

more complex than previously thought as shown by the presence of tricyclic diamondoid NAs in oil sands-influenced water (Rowland et al., 2011; 2012). Their presence of hydroxy acids (O_3 compounds) has also been demonstrated in oil sands-influenced water (West et al., 2013). The study at hand also demonstrated the presence of these oxidized NAs and showed that they are removed by the extraction process used for NAs. Chemistry results support varying levels of degradation that occur to NAs over long periods. NAs concentration was reduced by using microcosms by 64 – 74 % as well as reduced initial acute toxicity in rainbow trout (Toor et al., 2013). Despite rapid degradation, other NAs compounds remain recalcitrant for decades and it could be speculated that diamondoid-type structures could be resistant to bacterial degradation.

The results presented in this study demonstrates the immunotoxic effects of oil sands-influenced waters similar to those observed in yellow perch (Hogan et al., 2011; van den Heuvel et al., 2000) and caged rainbow trout (McNeil et al., 2012). The study also showed that extracted NAs conforming to the $C_nH_{2n+z}O_2$ formula are most likely not the cause of immunotoxicity found in the oil sands-influenced water. The exact compound(s) responsible for the effects seen in Demonstration Pond still remains to be determined. However, this study along with previous evidence would suggest that organic compounds are still the most likely candidates. Aromatic carboxylic acids, dibenzothiophenes, or oxidized NAs remain potential causative agents.

3.5 References

Arens, C.J., Hogan, N.S., Van Der Kraak, G.J., van den Heuvel, M.R., 2013. Sublethal effects of oil sands-affected water on white sucker (*Catostomus commersonii*). Environmental Toxicology and Chemistry submitted.

Clemente, J., Fedorak, P., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Frank, R.A., Kavanagh, R., Burnison, B.K., Headley, J.V., Peru, K.M., Der Kraak, G.V., Solomon, K.R., 2006. Diethylaminoethyl-cellulose clean-up of a large volume naphthenic acid extract. Chemosphere 64, 1346-1352.

Garcia-Garcia, E., Pun, J., Perez-Estrada, L.A., Din, M.G.-E., Smith, D.W., Martin, J.W., Belosevic, M., 2011. Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. Toxicology Letters 203, 62-73.

Government of Alberta. April 2011. Talk about oil sands. [online] http://www.energy.alberta.ca/ (Accessed January 2013).

Hagen, M.O., Garcia-Garcia, E., Oladiran, A., Karpman, M., Mitchell, S., El-Din, M.G., Martin, J.W., Belosevic, M., 2012. The acute and sub-chronic exposures of goldfish to naphthenic acids induce different host defense responses. Aquatic Toxicology 109, 143-149.

Han, X., MacKinnon, M.D., Martin, J.W., 2009. Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS. Chemosphere 76, 63-70.

Hogan, N.S., Groman, D., Phalen, L.J., van den Heuvel, M.R., 2011. Biochemical responses and disease incidence in yellow perch (*Perca flavescens*) exposed to oil sands process-affected waters. Platform presentation, Society of Environmental Toxicology and Chemistry, 32nd Annual Meeting, Boston, USA, November 13–17, 2011.

Holowenko, F.M., MacKinnon, M.D., Fedorak, P.M., 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry Water Research. 36,
2843-2855.

Inoue, T., Moritomo, T., Tamura, Y., Mamiya, S., Fujino, H., Nakanishi, T., 2002. A new method for fish leucocyte counting and partial differentiation by flow cytometry. Fish and Shellfish Immunology 13, 379–390.

Jivraj, M.N., MacKinnon, M., Fung, B., 1995. Naphthenic acid extraction and

quantitative analysis with FT-IR spectroscopy. In Syncrude Analytical Manuals, 4th ed. Research Department, Syncrude Canada Ltd., Edmonton, Alberta, Canada.

Jones, D., West, C.E., Scarlett, A.G., Frank, R.A., Rowland, S.J., 2012. Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract. Journal of Chromatography A 1247, 171-175.

Kavanagh, R.J., Burnison, B.K., Frank, R.A., Solomon, K.R., Van Der Kraak, G.J., 2009. Detecting oil sands process-affected waters in the Alberta oil sands region using synchronous fluorescence spectroscopy. Chemosphere 76, 120–126.

Kelly, E.N., Short, J.W., Schindler, D.W., Hodson, P.V., Ma, M., Kwan, A.K., Fortin, B.L., 2009. Oil sands development contributes polycyclic aromatic compounds to the Athabasca River and its tributaries. Proceedings of the National Academy of Sciences 106, 22346-22351.

Kelly, E.N., Schindler, D.W., Hodson, P.V., Short, J.W., Radmanovich,R., Nielsen,C.C., 2010. Oil sands development contributes elements toxic at low concentrations to the Athabasca River and its tributaries. Proceedings of the National Academy of Sciences 107, 16178–16183.

Köllner, B., Kotterba, G., 2002. Temperature dependent activation of leucocyte populations of rainbow trout, *Oncorhynchus mykiss*, after intraperitoneal immunisation with *Aeromonas salmonicida*. Fish & Shellfish Immunology 12, 35-48.

Logan, D.T., 2007. Perspective on Ecotoxicology of PAHs to Fish. Human and Ecological Risk Assessment 13, 302-316.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe, K.L., Phalen, L.J., Wagner, B.D., van den Heuvel, M.R., 2013. Immunotoxic effects of oil sands-derived naphthenic acids to rainbow trout. Aquatic Toxicology 126, 95-103.

MacKinnon, M.D., Boerger, H., 1986. Description of two treatment methods for detoxifying oil sands tailings pond water. Water Pollution Research Journal of Canada 21, 496-512.

McNeill, S.A., Arens, C.J., Hogan, N.S., Köllner, B., van den Heuvel, M.R., 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. Ecotoxicology and Environmental Safety 84, 254-261.

Mohamed, M.H., Wilson, L.D., Headley, J.V., Peru, K.M., 2008. Screening of oil sands naphthenic acids by UV–vis absorption and fluorescence emission spectrophotometry. Journal of Environmental Science and Health, Part A 43, 1700–1705.

Palmer, L.J., Hogan, N.S., van den Heuvel, M.R., 2012. Phylogenetic analysis and molecular methods for the detection of lymphocystis disease virus from yellow perch, *Perca flavescens* (Mitchell). Journal of Fish Diseases 35, 661-670.

Phalen, L.J., Köllner, B., Leclair, L.A., Hogan, N.S., van den Heuvel, M.R., 2013. The effects of benzo[a]pyrene on leukocyte distribution and antibody response in rainbow trout (*Oncorhynchus mykiss*). Toxicological Sciences submitted.

Rowland, S.J., Scarlett, A.G., Jones, D., West, C.E., Frank, R.A., 2011. Diamonds in the rough: identification of individual naphthenic acids in oil sands process water Environmental Science & Technology 45, 3154-3159.

Rowland, S.J., West, C.E., Scarlett, A.G., Ho, C., Jones, D., 2012. Differentiation of two industrial oil sands process-affected waters by two-dimensional gas chromatography/mass spectrometry of diamondoid acid profiles Rapid Communications in Mass Spectrometry 26, 572-576.

Sansom, B., Vo, N.T.K., Kavanagh, R., Hanner, R., MacKinnon, M., Dixon, D.G., Lee, L.E.J., 2013. Rapid assessment of the toxicity of oil sands process-affected waters using fish cell lines. *In Vitro* Cellular & Developmental Biology - Animal 49, 52-65.

Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Van Muiswinkel, W.B. (Ed.), Techniques in Fish Immunology. SOS Publications, Fair Haven, pp. 101–103.

Toor, N.S., Franz, E.D., Fedorak, P.M., MacKinnon, M.D., Liber, K., 2013. Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands. Chemosphere 90, 449-458.

van den Heuvel, M.R., Munkittrick, K.R., Stegeman, J.J., Dixon, D.G., 1995a. Second round interlaboratory comparison of hepatic ethoxyresorufin-O-deethylase activity in white sucker (*Catostomus commersoni*) exposed to bleached-kraft pulp mill effluent. Environmental Toxicology and Chemistry 14, 1513–1520.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Van Meer, T., Dobson, E.P., Dixon, D.G., 1999a. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). I. Water quality characteristics and yellow perch physiological and population responses. Canadian Journal of Fisheries and Aquatic Sciences 56, 1213-1225.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Dixon, D.G., 1999b. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). II. Chemical and biochemical indicators of exposure to oil sands related waters. Canadian Journal of Fisheries and Aquatic Sciences 56, 1226–1233.

van den Heuvel, M.R., Power, M., Richards, J., Mackinnon, M., Dixon, D.G., 2000. Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. Ecotoxicology and Environmental Safety 46, 334-341.

van den Heuvel, M.R., Hogan, N.S., Roloson, S.D., Van Der Kraak, G.J., 2012. Reproductive development of yellow perch (*Perca flavescens*) exposed to oil sands-affected waters. Environmental Toxicology and Chemistry 31, 654-662.

Wayland, M., Headley, J.V., Peru, K.M., Crosley, R., Brownlee, B.G., 2008. Levels of polycyclic aromatic hydrocarbons and dibenzothiophenes in wetland sediments and aquatic insects in the oil sands area of Northeastern Alberta, Canada. Environmental Monitoring and Assessment 136, 167–182.

West, C.E., Jones, D., Scarlett, A.G., Rowland, S.J., 2011. Compositional heterogeneity may limit the usefulness of some commercial naphthenic acids for toxicity assays. Science of the Total Environment 409, 4125-4131.

West, C.E., Scarlett, A.G., Pureveen, J., Tegelaar, E.W., Rowland, S.J., 2013. Abundant naphthenic acids in oil sands process-affected water: studies by synthesis, derivatisation and two-dimensional gas chromatography/high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry 27, 357-365.

Wilkie, M.P., Simmons, H.E., Wood, C.M., 1996. Physiological adaptations of rainbow trout to chronically elevated water pH (pH = 9.5). The Journal of Experimental Zoology 27 1-14.

CHAPTER 4

General Conclusion

4.1 Thesis Summary and Conclusions

The overarching hypothesis of this thesis was that naphthenic acids (NAs) extracted from oil sands-influenced waters will cause endocrine disruption and immunotoxic effects. The endocrine disrupting capabilities as well as estrogen and androgen binding capabilities were measured in Chapter 2 while immunotoxic effects in trout were measured in Chapter 3.

The first objective of this thesis (Chapter 2) was to use *in vitro* bioassays to determine if specific NAs fractions obtained from oil sands-influenced waters possess biological activity. The DCM NAs fraction elicited AhR-mediated activity after 24 h but not after 48 or 72 h indicating relatively easily metabolized compounds. The DCM fraction also had the highest affinity as an antagonist of estrogen and androgen receptors. By contrast corticosterone levels, as measured in the steroidogenesis assay, increased at the highest concentration of the main fraction.

The second objective of this thesis (Chapter 3) was to determine whether oil sands-influenced waters, and oil sands-derived NAs have the potential to be immunotoxic to rainbow trout in an *in vivo* laboratory exposure. Unlike the *in vitro* study, *in vivo* experiments could only be conducted with the main fraction where sufficient material existed for waterborne exposures. Oil sands-influenced waters and extracted NAs exposures resulted in an increase in bile fluorescence at phenanthrene wavelengths. Consistent with the *in vitro* study, liver CYP1A was not induced in those treatments. Trout in the oil sands-influenced water exposure showed a decrease in B- and T-

lymphocytes in blood as well as B-lymphocytes and myeloid cells in spleen and an increase in B-lymphocytes in head kidney. The extracted main NA fraction exposure showed no comparable immune cell response. There was a significant decrease in antibody production against *A. salmonicida* in both oil sands-influenced water exposures. Because oil sands-influenced waters affected multiple immune parameters, while extracted NAs impacts were limited, the NAs tested here are likely not the cause of immunotoxicity found in the oil sands-influenced water. The results presented in this study demonstrate the immunotoxic effects of oil sands-influenced waters similar to those observed in yellow perch (Hogan et al., 2011; van den Heuvel et al., 2000) and caged rainbow trout (McNeil et al., 2012). The study also showed that extracted NAs conforming to the $C_nH_{2n+z}O_2$ formula are most likely not the cause of immunotoxicity found in the oil sands-influenced water. The exact compound(s) responsible for the effects seen in Demonstration Pond still remains to be determined. However, this study along with previous evidence would suggest that organic compounds are still the most likely candidates.

4.2 Comparison Between *in vitro* and *in vivo* Studies

As part of a series of studies conducted since 1995, this research contributes to the endocrine and immunological impacts of oil sands-influenced waters as well as the effects of NAs derived from these waters. This is the first study to look at immunotoxicity of waterborne extracted NAs from oil sands-derived waters as well as endocrine disruption with specific extracted NAs fractions.

The main NAs did not induce CYP1A activation through AhR binding in both *in*

vitro and *in vivo* studies. The ^{1}H NMR results demonstrates that this fraction contained low intensity peaks around 6.8 – 8.2 ppm, which illustrates that these aromatic compounds have the potential to assume planar conformation and bind to the AhR. However, this was not observed in both exposures. Unlike in a previous investigation (McNeil et al., 2012), CYP1A induction was measured after a 21 d exposure of rainbow trout to Demonstration Pond. However, the DCM fraction was the only fraction to cause a CYP1A induction *in vitro* and it would be interesting to determine whether this fraction would trigger similar effects *in vivo*. Because this fraction can be found in Demonstration Pond used in the 7 d rainbow trout exposure, we would expect to see this induction transcend in the *in vivo* study. Perhaps a prolonged 21 d chronic exposure to the main NAs fraction and the oil sands-influenced waters in a laboratory setting would cause an induction in trout.

The increase in corticosterone levels when exposed to the main fraction may partly explain the immunosuppressive effects observed with the oil sands-influenced waters exposure. The immune response is typically inhibited by the hypothalamic-pituitary-adrenal (HPA) axis, specifically through the release several stress hormones such as glucocorticoids. Elevated levels of glucocorticoids inhibit the inflammatory response as well as the production of antibodies. Detrimental changes to the immune system include a decrease in lymphocytes populations, NK cell number and activity as well as a decrease in the ratio of helper to suppressor T-lymphocytes (Webster Markton and Glaster, 2008). These helper T-lymphocytes play an important role in adaptive immunity as well as B-lymphocyte antibody activation. A decrease in antibody production against *A. salmonicida* was observed herein as well as in a previous

investigation (McNeil et al., 2012). McNeil et al. 2012 also observed an increase in cortisol in fish exposed to Demonstration Pond. Stress through the action of hormones has detrimental effects on immunity and further investigations will be needed to understand the immunosuppressive effects of oil sands-influenced waters.

The DCM fraction containing neutral compounds has shown the most activity using *in vitro* methods. This fraction caused both an AhR induction, anti-estrogenic and anti-androgenic binding capabilities. This might give some insight to the endocrine disruption capabilities of oil sands-influenced waters found in previous investigations (Kavanagh et al., 2011, 2013; Lister et al., 2008; van den Heuvel et al., 1999b). Unfortunately, this fraction did not generate a large quantity from the extraction process and therefore cannot be used for *in vivo* investigations. However, the positive control, BaP, used for the *in vivo* 7 d exposure might elucidate results obtained from the DCM fraction *in vitro*. Further investigation into this fraction might explain the potential endocrine disrupting effects.

4.3 Future Directions

In vitro analyses provide immense data on the mechanisms underlying the whole body impacts we see in *in vivo* or field studies with oil sands-influenced waters. Because oil sands-influenced waters caused an immunotoxic effect in rainbow trout, the questions emerging from this study becomes which biochemical processes are involved and which compounds specifically alter the immune and reproductive-endocrine systems. As well, looking at endocrine disruption and the possible relationship that it may have to immunotoxicity is also important as the stress axis and immune function are highly

interconnected. Acute stress is thought to activate the innate stress response in fish while chronic stress suppresses the immune response and increases susceptibility to infection and disease (Tort, 2011). Exploring the potential link between these two systems will allow a better understanding of NAs effects on fish health. As well, an *in vivo* exposure using the DCM fraction might explain the immunotoxic effects observed in previous field experiments using oil sands-influenced waters (McNeil et al., 2012; van den Heuvel et al., 2000).

Identifying the exact compound(s) responsible for the biological effects observed is a daunting task. Significant work by Rowland and his team using comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCxGC/ToF-MS) has allowed a better understanding of the chemical nature of NAs. A more in-depth fractionation of the mixtures used in this study, with subsequent chemical characterization of the fractions might allow for the identification of specific compounds responsible for the effects observed in *in vitro* assessments. However, in many cases with complex mixture, such as the reproductive-endocrine activity of pulp mill effluent, the exact identity of bioactive agents defies identification even after decades of research. While such bioassay directed analysis is possible with the *in vitro* reproductive assays, due to the limited amount of material required in these assays, the absence of *in vitro* immuntoxicity tools make the determination of immune toxicants challenging. Even with fractionation as simple as was employed in this study, it was impossible to conduct *in vivo* immunotoxicology bioassays with all of the fractions.

With the Athabasca oil sands industry already reaching 176 km^2 of accumulated tailings and process-affected water bodies in the mineable area in 2010 (CAPP, 2013),

water remediation and reclamation has become critically important for environmental sustainability of the region. These waters need to be incorporated back into the environment once the oil from the area has all been mined out. Future investigations of the health of the ecosystem as well as understanding the compounds found in these waters would contribute substantially to answering questions regarding the toxicity of oil sands-influenced waters and their ability to support healthy fish populations.

4.4 References

Arens, C.J., Hogan, N.S., Van Der Kraak, G.J., van den Heuvel, M.R., 2013. Sublethal effects of oil sands-affected water on white sucker (*Catostomus commersonii*). Environmental Toxicology and Chemistry submitted.

Canadian Association of Petroleum Producers, 2013. The facts on: Oil sands.

Clemente, J., Fedorak, P., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Frank, R.A., Kavanagh, R., Burnison, B.K., Headley, J.V., Peru, K.M., Der Kraak, G.V., Solomon, K.R., 2006. Diethylaminoethyl-cellulose clean-up of a large volume naphthenic acid extract. Chemosphere 64, 1346-1352.

Han, X., MacKinnon, M.D., Martin, J.W., 2009. Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS. Chemosphere 76, 63-70.

Hogan, N.S., Groman, D., Phalen, L.J., van den Heuvel, M.R., 2011. Biochemical responses and disease incidence in yellow perch (*Perca flavescens*) exposed to oil sands process-affected waters. Platform presentation, Society of Environmental Toxicology and Chemistry, 32nd Annual Meeting, Boston, USA, November 13–17, 2011.

He, Y., Wiseman, S.B., Zhang, X., Hecker, M., Jones, P.D., Gamal El-Din, M., Martin, J.W., Giesy, J.P., 2010. Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line. Chemosphere 80, 578-584.

He, Y., Wiseman, S.B., Hecker, M., Zhang, X., Wang, N., Perez, L.A., Jones, P.D., Gamal El-Din, M., Martin, J.W., Giesy, J.P., 2011. Effect of ozonation on the estrogenicity and androgenicity of oil sands process-affected water. Environmental Science & Technology 45, 6268-6274.

Jones, D., West, C.E., Scarlett, A.G., Frank, R.A., Rowland, S.J., 2012. Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract. Journal of Chromatography A 1247, 171-175.

Kavanagh, R.J., Frank, R.A., Oakes, K.D., Servos, M.R., Young, R.F., Fedorak, P.M., MacKinnon, M.D., Solomon, K.R., Dixon, D.G., Van Der Kraak, G., 2011. Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters. Aquatic Toxicology 101, 214-220.

Kavanagh, R.J., Frank, R.A., Burnison, B.K., Young, R.F., Fedorak, P.M., Solomon, K.R., Van Der Kraak, G., 2012. Fathead minnow (*Pimephales promelas*)

reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology 116-117, 34-42.

Kavanagh, R.J., Frank, R.A., Solomon, K.R., Van Der Kraak, G., 2013. Reproductive and health assessment of fathead minnows (*Pimephales promelas*) inhabiting a pond containing oil sands process-affected water. Aquatic Toxicology 130-131, 201-209.

Lister, A., Nero, V., Farwell, A., Dixon, D.G., Van Der Kraak, G., 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Aquatic Toxicology 87, 170-177.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe, K.L., Phalen, L.J., Wagner, B.D., van den Heuvel, M.R., 2013. Immunotoxic effects of oil sands-derived naphthenic acids to rainbow trout. Aquatic Toxicology 126, 95-103.

McNeill, S.A., Arens, C.J., Hogan, N.S., Köllner, B., van den Heuvel, M.R., 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. Ecotoxicology and Environmental Safety 84, 254-261.

Rowland, S.J., Scarlett, A.G., Jones, D., West, C.E., Frank, R.A., 2011a. Diamonds in the rough: identification of individual naphthenic acids in oil sands process water. Environmental Science & Technology 45, 3154-3159.

Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., Frank, R.A., 2011b. Identification of individual tetra- and pentacyclic naphthenic acids in oil sands process water by comprehensive two- dimensional gas chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 25, 1198–1204.

Rowland, S.J., West, C.E., Jones, D., Scarlett, A.G., Frank, R.A., Hewitt, L.M., 2011c. Steroidal aromatic 'naphthenic acids' in oil sands process-affected water: structural comparisons with environmental estrogens. Environmental Science & Technology 45, 9806-9815.

Rowland, S.J., West, C.E., Scarlett, A.G., Ho, C., Jones, D., 2012. Differentiation of two industrial oil sands process-affected waters by two-dimensional gas chromatography/mass spectrometry of diamondoid acid profiles. Rapid Communications in Mass Spectrometry 26, 572-576.

Tetrault, G.R., McMaster, M.E., Dixon, D.G., Parrott, J.L., 2003. Using reproductive endpoints in small forage fish species to evaluate the effects of Athabasca oil sands activity. Environmental toxicology and chemistry 22, 2275-2782.

Tort, L., 2011. Stress and immune modulation in fish. Developmental & Comparative Immunology 35, 1366-1375.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Dixon, D.G., 1999b. Effects of oil sands related aquatic reclamation on yellow perch (*Perca flavescens*). II. Chemical and biochemical indicators of exposure to oil sands related waters. Canadian Journal of Fisheries and Aquatic Sciences 56, 1226-1233.

van den Heuvel, M.R., Hogan, N.S., Roloson, S.D., Van Der Kraak, G.J., 2012. Reproductive development of yellow perch (*Perca flavescens*) exposed to oil sands-affected waters. Environmental Toxicology and Chemistry 31, 654-662.

van den Heuvel, M.R., Power, M., Richards, J., Mackinnon, M., Dixon, D.G., 2000. Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. Ecotoxicology and Environmental Safety 46, 334-341.

Webster Marketon, J.I., Glaser, R., 2008. Stress hormones and immune function. Cellular Immunology 252, 16-26.

West, C.E., Scarlett, A.G., Pureveen, J., Tegelaar, E.W., Rowland, S.J., 2013. Abundant naphthenic acids in oil sands process-affected water: studies by synthesis, derivatisation and two-dimensional gas chromatography/high-resolution mass spectrometry. Rapid Communications in Mass Spectrometry 27, 357-365.